The Regulation of Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) in the Gastric Epithelium

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by Angharad Wyn Michael

September 2008

Contents

Acknowledgements

I would like to thank my supervisors Andrea Varro and Rod Dimaline for their help, support and guidance throughout the past three years.

I also thank Dr. Keith Bodger for supplying immunohistochemistry images.

Thank you to everyone I've had the pleasure of working with in Green Block over the last three years, for help and support in the lab, and also for the good times outside the lab. It has been a privilege to be part of the lab.

I thank my friends for keeping me sane during my PhD and cheering me up when things were going badly, also for the emails which brightened up my days.

Hoffwn ddiolch i'm rhieni, Iorwerth a Medi Michael, am eu cymorth a chefnogaeth dros y bedair blynedd ddiwethaf. Diolch am eich ffydd y byddwn yn llwyddiannus, ac am bob amser fod yn barod i wrando.

Last but not least I'd like to thank Gareth for his support during my PhD. Thank you for listening to all my worries and making me feel better, and helping me to relax.

List of Figures

List of Tables

List of Abbreviations

α-SMA – α-Smooth muscle actin

ADAM - A disintegrin and a metalloproteinase domain

ADAMT - ADAM with thrombospondin like repeats

BSA - Bovine serum albumin

CAG – Chronic atrophic gastritis

COX -2 - Cyclooxygenase-2

ECL cell - Enterochromaffin-like cell

ECM - Extracellular matrix

EGF - Epidermal growth factor

ERK - Extracellular signal-related kinases

FBS - Fetal bovine serum

FITC - Fluorescein isothiocyanate

FSM - Full serum medium

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

HB-EGF - Heparin-binding EGF-like growth factor

HGF - Hepatocyte growth factor

HRP - Horseradish peroxidase

IGF-II - Insulin-like growth factor-II

IGFBP-5 - Insulin-like growth factor binding protein-5

IHC - Immunohistochemistry

JNK - Jun amino-terminal kinases

LB - Luria-Bertani broth

MAPK - Mitogen activated protein kinases

MEK - Mitogen-activated protein kinase kinase

MMP - Matrix metalloprotinases

MT-MMP - Membrane type MMP

PA - Pernicious anaemia

PBS - Phosphate buffered saline

PDGF - Platelet derived growth factor

PI3K - Phosphoinositide-3 kinase

PKC - Protein kinase C

PMA - Paramethoxyamphetamine

PPIs - Proton pump inhibitors

Q-PCR - Quantitative polymerase chain reaction

SDS - Sodium dodecyl sulfate

SDW – Sterile distilled water

SFM - Serum free medium

TGF-α - Transforming growth factor-α

TGF-β - Transforming growth factor-β

TIMP - Tissue inhibitors of matrix metalloprotinases

uPA - Plasminogen activator

VEGF - Vascular endothelial growth factor

Publications and Presentations

Publications

Bodger K, Ahmed S, Pazmany L, Pritchard DM, Michael A, Khan AL, Dimaline R, Dockray GJ, Varro A. Altered gastric corpus expression of tissue inhibitors of metalloproteinases in human and murine Helicobacter infection. *J Clin Pathol.* (2008) **61** (1) 72-8.

Presentations

Wellcome Trust PhD conference – January 2008. Poster presentation

Bristol-Liverpool Symposium – September 2006. Oral presentation

Liverpool University Research day – March 2007. Poster presentation

Bristol-Liverpool Symposium – September 2007. Oral presentation

Abstract

The extracellular matrix (ECM) is constantly remodelled in healthy tissues; this is essential in normal physiological processes such as wound healing. Remodelling is regulated by the balanced activities of proteases and their inhibitors. An imbalance in activity can result in pathology such as fibrosis and cancer.

The proteases mainly responsible for breakdown of the ECM are the matrix metalloproteinases (MMPs). They are important for normal maintenance of the ECM, but also have roles in pathophysiology.

The tissue inhibitors of matrix metalloproteinases (TIMPs) are the main specific inhibitors of MMP activity. There are 4 identified human TIMPs, TIMP 1-4. Interestingly, TIMPs have roles that are independent of MMP inhibition, such as promoting cell proliferation and migration. Increased levels of TIMPs have been found in several types of cancers, including gastric cancer.

It has been found in our laboratory and by others that certain stimuli, such as *Helicobacter pylori* infection, and the gastric hormone gastrin, increase the expression of MMPs in the gastric epithelium, which is associated with pathology including the development of gastric carcinoma. The aim of this work was to investigate the regulation of TIMPs in the gastric epithelium.

H. pylori infection caused upregulation of TIMP-1, 3 and 4 in human gastric biopsy samples. Similar results were obtained in mice infected with *H. felis.* Increased expression was also seen in gastric biopsies from patients with pathologically elevated circulating gastrin concentrations.

TIMP 1 and 3 are expressed both in epithelial cells and in gastric myofibroblasts, TIMP-4 expression was restricted to epithelial cells. TIMP-1, 3 and 4 are also expressed in the gastric cancer cell line, AGS-G_R. Stimulation with gastrin or EGF increased expression of TIMP-1, 3 and 4 luciferase-reporter constructs in AGS-G_R cells. Inhibiting MEK1/2 markedly reduced, and inhibiting PKC partially inhibited the gastrin response of the TIMP-3-luc construct.

Stimulation of AGS-G_R cells with G17 also induced c-fos expression, and co-transfection of AGS-G_R cells with TIMP-3-luc and c-fos resulted in increased luciferase activity to a comparable level to that seen with G17 stimulation.

TIMP-3 also inhibited gastrin-induced migration of AGS-G_R cells.

In conclusion, I have shown that TIMP expression is affected by *Helicobacter* infection and elevated gastrin, TIMPs are expressed in a cell specific manner, and TIMP-3 expression is induced in AGS-G_R cells by activation of the MAPK pathway via AP1. The results suggest that TIMPs have an important role in gastric remodelling during both health and disease.

Chapter 1

Introduction

1.1 Epithelial organisation in the stomach

The human gastric mucosa consists of a complex epithelium, lamina propria and a thin layer of smooth muscle called the muscularis mucosa. The epithelium is folded into tubular gastric glands that extend into the muscularis mucosa. The glands are divided into isthmus, neck and base regions, and contain a variety of different specialised cell types, that exist in characteristic locations. Surface mucus and mucus neck cells are typically present in the isthmus and neck region of the gland. Acid secreting parietal cells are mostly found in the neck region, and histamine producing enterochromaffin-like (ECL) cells, gastrin producing G-cells, somatostatin producing D-cells and pepsinogen secreting chief cells are found towards the base of the gland (Fig 1).

Parietal and ECL cells exist mainly in glands located in the corpus of the stomach, while the glands in the antrum contain more G-cells, D-cells and mucus cells.

The cells of the gastric glands all originate from a single pool of progenitor cells located in the neck region of the gland, these cells migrate upwards and downwards along the gland.

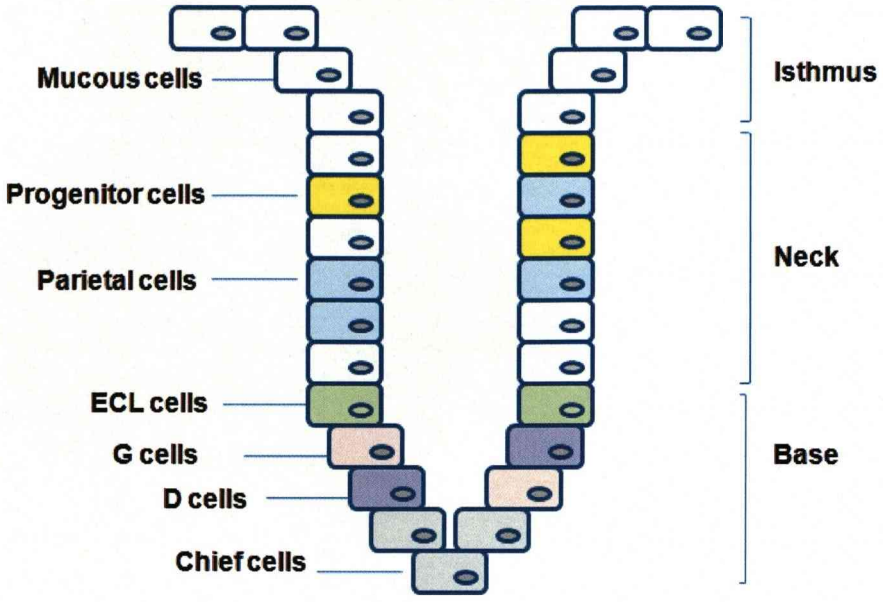


Fig 1: Representation of a gastric gland showing locations of cells. Glands in the corpus region of the stomach would normally contain parietal, ECl and chief cells, while glands in the antrum contain G-cells and pepsinogen containing cells, parietal cells are rarely found in the antrum.

1.2 Gastrin

It has long been established that food stimulates the secretion of gastric acid in the stomach (Beaumont 1833). It was thought that nervous mechanisms were responsible for this until the discovery of the hormone secretin, which stimulates pancreatic secretion, by Bayliss and Starling in 1902. This invited the question of whether gastric acid secretion could also be controlled by a hormonal mechanism.

Gastrin was discovered in 1905, and was shown to directly stimulate gastric acid secretion (Edkins 1905). Gastrin was isolated from gastric antrum and characterised by Gregory and Tracy (1964), who showed that the gastrins were 17 and 34 amino acid C-terminal amidated peptides, which they described as G17 and G34.

The gastrin precursor, preprogastrin is cleaved to give progastrin. Progastrin is cleaved by subtilisin-like prohormone convertases and carboxypeptidase E, generating COOH-terminal Gly-gastrins (G34-Gly and G17-Gly). These "non-classical gastrins" are converted to the corresponding COOH-terminally amidated peptides (G34 and G17), known as "classical gastrins" (Dockray 2004).

1.2.1 Actions of Gastrin

It is well-established that gastrin, released from antral G-cells, mediates increased gastric acid secretion after food intake (Walsh 1994). Gastrin acts via the CCK2 receptor (CCK2R) (Dufrense *et al.* 2006) which is mainly located on the parietal and ECL cells in the gastric epithelium (Kopin *et al.* 1992, Dockray *et al.* 2005). Gastrin acts by stimulating ECL cells to release histamine, this then acts as a paracrine mediator stimulating parietal cells to release acid. As acid concentrations increase, there is feedback on antral D-cells resulting in the release of somatostatin, thus inhibiting gastrin release, as shown in Fig 2. (Hersey and Sachs 1995, Dockray *et al.* 2001).

Gastrin also has a role in regulating ECL cell numbers, and regulates the expression of ECL genes such as histidine decarboxylase (HDC) and vesicular monoamine

transporter type 2 (VMAT-2), which are responsible for histamine synthesis and storage (Dockray *et al.* 2001).

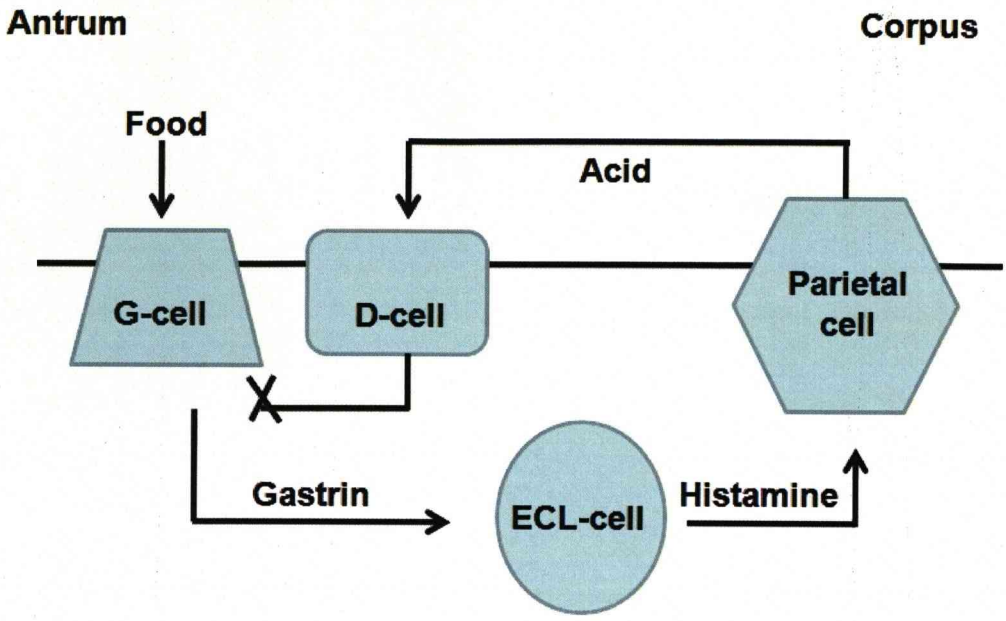


Fig 2: A schematic representation of acid release by gastrin in the stomach. G-cells release gastrin in response to food intake, gastrin causes ECL cells to release histamine which stimulates acid secretion from parietal cells, this feeds back on D-cells which release somatostatin to inhibit further gastrin release.

Elevated gastrin can stimulate the proliferation of gastric epithelial cells, and increase the numbers of parietal cells and ECL cells (Jensen 2002). Gastric epithelial progenitor cells are not thought to express the CCK2-receptor, so these responses to gastrin are likely to be due to the release of growth factors released by the parietal or ECL cells (Dockray *et al.* 2001).

Activation of the CCK2-receptor is linked to a wide range of biological responses such as stimulation of migration and invasion, and inhibition of apoptosis (Todisco

et al. 2001, Wroblewski *et al.* 2002, Noble *et al.* 2003). These processes are linked to the oncogenic properties of gastrin.

Recent gene array studies have identified many previously unknown targets of gastrin, such as plasminogen activator inhibitor (PAI)-2, MMP-7 and -9 and TIMP-3 (Varro *et al.* 2002 b). This is interesting because it suggests roles for gastrin in epithelial organisation, in health and disease.

1.2.2 Hypergastrinaemic conditions

1.2.2.1 Gastrinoma

Gastrinomas are gastrin secreting tumours normally found in the pancreas or duodenum. They may be sporadic, or occur on a background of multiple endocrine neoplasia type-1 (MEN-1). Plasma gastrin concentration is usually elevated to the 50-10,000pM range in gastrinoma patients (normal fasting gastrin concentration <50pM), and is also accompanied by high basal acid release. Approximately 30% of patients with gastrinoma and MEN-1 develop ECL carcinoid tumours (Gibril *et al.* 2004), although in patients with sporadic gastrinoma the incidence of ECL carcinoid tumours is less than 1% (Peghini *et al.* 2002).

1.2.2.2 Achlorhydric conditions

When acid production is inhibited, circulating gastrin concentrations increase. This is because acid release normally inhibits G-cell function by causing D-cells to release somatostatin which acts as a paracrine inhibitor of G-cell function. In pernicious

anaemia (PA) achlorhydria, due to loss of parietal cells, is present over many years, and this leads to high plasma gastrin concentrations of up to 2000pM. 5-10% of patients with PA develop ECL cell carcinoid tumours (Bordi *et al.* 1991, Bordi *et al.* 1995), which in rare cases can become large and carry a significant risk of metastasis (Thomas *et al.* 1995).

1.2.2.3 Helicobacter pylori infection

Infection with the bacterium *Helicobacter pylori* is associated with increased plasma gastrin concentration (Levi *et al.* 1989). One of the factors causing the elevated gastrin is the effect of proinflammatory cytokines acting directly on the G-cells, and possibly on the D-cells to inhibit somatostatin release, which also contributes to elevated gastrin. *Helicobacter pylori* produces ammonia and this results in a local increase in pH, which also reduces somatostatin release by the D-cells (Calam *et al.* 1997).

When *Helicobacter pylori* infection is present in the antrum only, increased gastrin secretion leads to increased acid production, often resulting in peptic ulcers (Calam *et al.* 1997). When the infection is present in both the antrum and corpus, inflammation can lead to atrophic gastritis, which is linked to a further increase in plasma gastrin concentration due to reduced acid inhibition of gastrin release by the G-cells (McColl *et al.* 2000). (See also section 1.4.1).

1.2.3 Ins-GAS mice

Ins-GAS mice are an animal model of hypergastrinaemia. They contain a transgene in which a rat insulin promoter drives a human gastrin minigene to produce amidated gastrin in pancreatic β-cells. The mice have raised plasma gastrin concentrations. They show increased proliferation in the gastric mucosa and, after 4 months of age, loss of parietal cells with subsequent decrease in acid secretion. The mice develop hyperplasia, similar to that seen in humans with chronic atrophic gastritis. Older mice have an increased tendency to develop gastric cancer. The mice can be infected with *Helicobacter*, and this increases the rate of development of gastric cancer (Wang *et al.* 1996, Wang *et al.* 2000).

1.2.4 AGS cells

AGS cells are a human gastric cancer cell line, that do not express the gastrin-CCK2 receptor. AGS-G_R cells are AGS cells stably transfected with a vector encoding the human CCK2R and thus respond to gastrin (Watson *et al.* 2001). Gastrin upregulates expression of many genes in this cell line, for example plasminogen activator inhibitor (PAI)-2 and TIMP-3 (Varro *et al.* 2002).

1.2.5 Gene expression regulatedby gastrin

Amidated gastrins can increase the expression of many growth factors such as TGF-α, HB-EGF and amphiregulin (Tsutsui *et al.* 1997), and in Ins-GAS mice expression of TGF-α and HB-EGF in the stomach are increased (Wang *et al.* 2000).

Parietal cells can release HB-EGF in the stomach, and it is thought that gastrin leads to EGFR mediated autocrine inhibition of parietal cell proliferation and paracrine inhibition of mucous cell numbers, thus controlling cell numbers and development in the gastric gland (Wang *et al.* 2000).

Gastrin can also affect the expression of Reg in the gastric mucosa. Reg1α is expressed in ECL and chief cells in the human stomach, and mutations of Reg1α are often present in ECL carcinoid tumours, suggesting a role for Reg1α as a tumour suppressor (Higham *et al.* 1999).

1.3 Extracellular Protease systems

The breakdown of the extracellular matrix (ECM) is an essential part of many normal processes such as embryonic development, morphogenesis, reproduction and tissue remodelling. This is also important in disease conditions such as gastric ulcer, inflammatory diseases and also cancer, where processes such as local invasion, angiogenesis and vascular invasion all contribute to disease progression (Nagase and Woessner 1999, Sternlicht and Werb 2001).

1.3.1 Matrix metalloproteinases (MMPs)

The proteinases mainly responsible for the breakdown of the ECM are the matrix metalloproteinases (MMPs). The MMPs are a zinc-dependent family of at least 23 extracellular proteinases capable of degrading the structural proteins of the ECM and are involved in remodelling of the extracellular matrix. MMPs are crucial in several physiological processes such as embryonic development, tissue morphogenesis and wound healing (Sternlicht and Werb 2001), moreover levels of MMPs are often increased in disease. MMP overexpression is linked to matrix degradation in emphysema, aortic aneurysms, and many inflammatory conditions (Ashworth *et al.* 1999, Curci *et al.* 1998, McCawley and Matrisian 2001).

There is also extensive evidence that MMPs contribute to cancer development, the role of MMPs in cancer was recognised in the early 1980s with the realisation that proteolysis was essential for tumour invasion, and the discovery of a type IV collagenase involved in melanoma invasion and metastasis (Liotta *et al.* 1980). MMPs are normally expressed by stromal cells, rather than the tumour cells themselves. MMP expression by stromal cells such as connective tissue cells, including fibroblasts and inflammatory cells, is thought to be induced in response to the tumour cells (Basset *et al.* 1990, Nabeshima *et al* 1991). MMPs contribute to cancer progression by altering the extracellular environment. In order for tumours to become invasive and metastasise they must be able to first break through the basement membrane, invade through local connective tissue and metastasise to distant organs (Kleiner 1999). MMPs are important in this process of malignancy as they break down the ECM, allowing invasion and thus spread of cancer cells throughout the body (Sternlicht and Werb 2001). They can also contribute to tumour

progression by promoting invasion, angiogenesis and metastasis (Powell *et al.* 1993, Kim *et al.* 1998, Itoh *et al.* 1998, Zhai *et al.* 2005).

MMPs are implicated in the progression of oral squamous cell carcinoma, colorectal cancer, gastric cancer, ovarian cancer and cervical cancer, to name a few, with overexpression often indicating a worse prognosis (Baker *et al.* 2005, Islekel *et al.* 2007, Kitoh *et al.* 2004, Kenny *et al.* 2008, Zhai *et al.* 2005).

Increased expression of MMP-9 enhances invasion of the ECM by AGS cells, a human gastric cancer cell line. Also increased expression of MMP-9 was found in the stomach and ECL carcinoid tumours of patients with elevated gastrin concentrations (Wroblewski *et al.* 2002).

1.3.1.1 Regulation of MMPs

It is important that the activity of MMPs is tightly controlled, because of their activity in breaking down the ECM, and the danger that unregulated digestion can destroy tissues. The MMPs are regulated in several ways. Most MMPs are secreted from the cell as inactive proforms, and their activation is an important step in regulating MMP activity outside the cell (Kleiner 1999). The expression of MMP mRNA is tightly controlled, individual MMPs are expressed by specific cell types, at low levels, and expression increases only in situations where MMP activity is required such as inflammation and wound healing (Coussens *et al.* 2001).

The main specific inhibitors of MMP activity are the tissue inhibitors of matrix metalloprotinases (TIMPs).

1.3.2 Tissue inhibitors of matrix metalloprotinases (TIMPs)

Four homologous human TIMPs have been identified, TIMPs 1-4. The TIMPs inhibit MMPs through the formation of reversible 1:1 stoichiometric complexes, which are resistant to heat denaturation and proteolytic degradation (Gomez *et al.* 1997).

The TIMPs are small proteins, about 21-28KDa in size. They have similar amino acid sequences, a conserved gene structure and 12 conserved cysteine residues forming 6 disulfide bridges, folding the protein into an N terminal and C terminal domain. The N terminal domain of the TIMPs is the most highly conserved between the four, and it is this part that is responsible for MMP inhibition (Caterina *et al.* 1997), the C terminal domain is more likely to mediate effects independent of MMP inhibition (Chirco *et al.* 2006).

Unlike the MMPs, the TIMPs are all secreted from the cell in an active form (Sternlicht and Werb 2001). All the TIMPs have similar activities in binding to MMPs, however they do differ in their affinities to the various MMPs. TIMP-1 preferentially binds to MMPs 1, 3 and 9, TIMP-2 to MMP-2, TIMP-3 to MMP-2 and -9, and no specific inhibition has yet been discovered for TIMP-4 (Lambert *et al.* 2004). The TIMPs also differ in their abilities to bind to the inactive proMMPs, for example TIMP-3 will bind to proMMP-2 and -9 and TIMP-2 and -4 will bind proMMP-2. TIMP-2 and -3 have also been found to bind membrane type MMPs, while the other TIMPs do not (Lambert *et al.* 2004).

TIMP-3 is unique in that it is the only TIMP able to bind to the ECM. It was first discovered as an ECM bound molecule in chick embryo fibroblasts undergoing transformation (Blenis and Hawkes 1984). TIMP-3 binds to the ECM by association

between its N-terminal domain and heparan sulphate and chondroitin sulphate chains of proteoglycans on the cell surface (Yu *et al.* 2000). Here it can regulate movement through the basement membrane and stroma.

TIMP-1 and -3 inhibit the ADAMs (A Disintegrin and A Metalloproteinase domain) and the ADAMTSs (ADAM with Thrombospondin like repeats), as well as the MMPs. TIMP-1 inhibits ADAM-10 and TIMP-3 inhibits ADAMs 12, 17, 19, 10, and ADAMTs 4 and 5 (Amour *et al.* 2000, Baker *et al.* 2002, Kashiwagi *et al.* 2001).

The TIMPs are expressed by many cell types, although at low levels in normal tissues (Lambert *et al.* 2004), TIMP-2 expression is constitutive and widely expressed throughout the body, while the expression of TIMP-1, -3 and -4 is inducible and more tissue specific. TIMP-1 is expressed highly in the reproductive organs, TIMP-3 in the heart, kidney and thymus, and TIMP-4 in the brain, heart, ovary and skeletal muscle (Leco *et al.* 1997).

It is thought that TIMPs have roles in the activation of some MMPs, for example, formation of a complex between MT1-MMP, proMMP-2 and TIMP-2 is essential for proMMP-2 activation by MT1-MMP on the cell surface (Itoh *et al.* 2006).

In normal tissues the TIMPs and MMPs exist in a delicate balance and the digestion of the ECM is tightly regulated (Woessner 2001). As a simple model, it is thought that as the ratio of active MMPs to TIMPs increases, there is a destruction of the tissues and tumour cells have an increased metastatic potential (Kleiner 1999). An increase in active MMP production often occurs simultaneously with increased TIMP expression; however MMPs must be produced in excess of TIMPs in order to be able to degrade the ECM (Bergin *et al.* 2004).

1.3.2.1 Roles independent of MMP inhibition

In addition to their roles in the inhibition of MMPs, TIMPs have other various biological activities, independent of their MMP inhibitory activity (Sternlicht and Werb 2001). TIMP-1 was first identified as an erythroid potentiating protein, and as a protein involved in stimulating the growth of some cell lines (Gasson *et al.* 1985, Bertaux *et al.* 1991). It has also been found that TIMP-2 has erythroid potentiating activity, and stimulates the growth of lymphoma cells (Stetler-Stevenson *et al.* 1992, Hayakawa *et al.* 1994).

TIMPs that are mutated and lack MMP inhibitory activity have been shown to retain their growth promoting activity (Hayakawa *et al.* 1994, Wingfield *et al.* 1999). Also synthetic MMP inhibitors do not always abolish the effect of TIMPs on processes such as growth promotion, suggesting roles for TIMPs in these processes that are unrelated to MMP inhibition (Guedez *et al.* 1998).

NMR and crystallography studies of TIMP-1 have shown that it's MMP inhibitory and growth promoting activities are physically and functionally distinct (Chesler *et al.* 1995).

This could explain the paradox whereby TIMPs are often overexpressed in cancer, and that instead of being beneficial, this is often associated with poor prognosis (Guedez *et al.* 1998, Joo *et al.* 2000, Islekel *et al.* 2006).

1.3.2.2 TIMPs in disease

TIMPs are found to be overexpressed in several diseases, and this has been linked to both poor or good outcomes. The different TIMPs seem to have different roles, and to be regulated differently in different cells. TIMPs can also have contradictory influences on cellular processes depending on the cell type.

TIMP-1 has been found to increase the proliferation of various tumour cells (Hayakawa *et al.* 1992). It is involved in Burkitt's lymphoma, where is inhibits the apoptosis of the lymphoma cells, thus worsening prognosis (Guedez *et al.* 1998), laryngeal squamous cell carcinoma (Gorogh *et al.* 2006), and gastric carcinoma biopsies (Koyama *et al.* 2004, Kubben *et al.* 2006), where it is associated with reduced survival (Joo *et al.* 2000, Yoshikawa *et al.* 2001). High levels of TIMP-1 were found in bone metastasis from breast cancer (Voorzanger-Rousselot *et al.* 2006), and also in poorly differentiated colorectal cancers, suggesting a negative contribution of TIMP-1 in tumour differentiation and prognosis in this type of cancer (Islekel *et al.* 2006). TIMP-1 overexpression is associated with a poor prognosis in many other types of cancer including breast, lymphoma and non small cell lung cancer (Chirco *et al.* 2006).

Overexpression of TIMP-1 was found in fibrotic Crohn's disease, indicating that TIMP-1 may play a part in the progression of this inflammatory bowel disease, possibly through the inhibition of MMP activity (McKaig *et al.* 2003).

TIMP-2 stimulates the invasion and metastasis of colorectal, lung and breast cancers (Kossakowaska *et al.* 1996, Porter *et al.* 2004), but can inhibit invasion in various cancer cell lines (Baker *et al.* 1999). However, overexpression of TIMP-2 was found to lower metastatic potential in oral squamous cell carcinoma (Baker *et al.* 2005). TIMP-2 stimulates apoptosis in colorectal cancer cells, smooth muscle cells and retinal pigmented epithelial cells, but inhibits apoptosis in other various cancer cell lines (Brand *et al.* 2000, Valente *et al.* 1998, Woessner 2001). TIMP-2 inhibits angiogenesis of endothelial cells, independent of MMP inhibition (Seo *et al.* 2003). Koyama *et al.* 2004 found increased expression of TIMP-2 in gastric carcinoma cells.

TIMP-2 has also been described as inhibiting proliferation of several cell types including endothelial cells, renal cancer cells and glioma cells, thus having an inhibitory effect on cancer progression (Murphy *et al.* 1993, Miyake *et al.* 1999, Bello *et al.* 2001).

TIMP-3 is the only TIMP directly related to a disease. Mutations of certain tyrosine and cysteine residues on the *TIMP-3* gene are present in Sorsby's fundus dystrophy. This is an autosomal dominant macular disorder that leads to progressive loss of sight, eventually resulting in blindness. Excessive deposition of material within Bruch's membrane is thought to be a direct consequence on the influence of TIMP-3 on the ECM (Felbor 1995).

Decreased expression of TIMP-3 was found to be associated with increased invasiveness in oesophageal cancer cells (Miyazaki *et al.* 2004).

There is evidence that TIMP-3 is involved in inducing apoptosis of smooth muscle cells and neuronal cells, and is involved in the progression of neurodegenerative

diseases such as Alzheimer's disease (Baker *et al.* 1998, Lee *et al.* 2008). TIMP-3 overexpression is also linked to the progression of idiopathic pulmonary fibrosis (Garcia-Alvarez *et al.* 2006)

TIMP-4 inhibits proliferation of Wilm's tumour cells (Celiker *et al.* 2001). Also it stimulates apoptosis in cardiac fibroblasts, but inhibits apoptosis in breast cancer (Baker *et al.* 2002).

Overexpression of TIMPs was found in Peyronie's disease, a fibrotic condition of the tunica albuginea. TIMP protein accumulated in the fibrotic lesions, and there was also decreased local MMP activity (Del Carlo *et al.* 2008). Overexpression of TIMP-1 mRNA has been found in fibrotic kidneys, and TIMP-1, -2 and -4 expression was increased in cardiac fibrosis (Mozes *et al.* 1999, Seeland *et al.* 2002).

Increased levels of TIMP-2 and TIMP-4 have been found in gastric carcinoma biopsies (Koyama *et al.* 2004a, Kubben *et al.* 2006), this is associated with reduced survival (Joo *et al.* 2000, Yoshikawa *et al.* 2001). TIMPs can affect cell growth and survival of gastric cancer cells independently of their roles in MMP activation and inhibition (Koyama *et al.* 2004).

1.3.2.3 TIMP knockout mice

TIMP -1, -2 and -3 null mice have been described, these are all viable and fertile. However TIMP-3 null mice show spontaneous lung airspace enlargement at around 14 days after birth, and the animals usually die at about 1 year old (Leco *et al.* 2001). This is due to a disturbance of the MMP/TIMP balance, with an increase in MMP activity, resulting in increased turnover of collagen in the alveoli, and a reduction in epithelial cell proliferation (Gill *et al.* 2003). In normal lung development TIMP-3 restricts MMP activity resulting in ECM cell signalling and proliferation of epithelial cells in the developing bronchioles (Gill *et al.* 2006).

TIMP-2 null mice show motor deficits and neuromuscular junction alterations (Jaworski *et al.* 2006). TIMP-1 null mice exhibit alterations in the reproductive system, including altered uterine morphology, increased MMP activity in the uterus and a reduction of reproductive lifespan in female mice (Nothnick *et al.* 2000, Nothnick *et al.* 2001a, Nothnick *et al.* 2001b).

1.3.2.4 Synthetic MMP inhibitors

Several synthetic MMP inhibitors have been developed, however clinical trials have mostly been unsuccessful. Batimastat was one of the first to be clinically tested. It was found to inhibit tumour growth and metastasis (Macauley *et al.* 1999). However side effects included musculoskeletal pain and inflammation. Trials with other synthetic inhibitors have also been disappointing due to their lack of effect on human cancer progression or survival rate (Brown 2000, Bramhall *et al.* 2001). Clinical

trials with tanomastat in pancreatic cancer were terminated due to poorer survival rates in the treatment group compared to the control (Moore *et al.* 2001).

Batimastat treatment caused increased liver metastasis in nude mice, and liver specific overexpression of MMP-2 and -9, angiogenesis factors and caspase-1, even in tumor-free animals (Kruger *et al.* 2001). MMP inhibitors causing a reduction in plasma levels of MMP-9 in mice led to increased tumor growth and vascularisation (Pozzi *et al.* 2002).

These types of side effects need to be considered, and further development is needed before clinical use of synthetic MMP inhibitors.

1.4 *Helicobacter pylori* infection

Helicobacter pylori is a member of a family of bacteria that colonise the mammalian stomach, it was discovered in the human stomach in 1982 by Marshall and Warren. The bacterium is associated with gastritis and peptic ulcer, and a higher incidence of gastric cancer has been reported with *H. pylori* infection (Parsonnet *et al.* 1991, Forman *et al.* 1991, Uemura *et al.* 2001), The World Health Organization and the International Agency for Research on Cancer classified *H. pylori* as a class I carcinogen in 1994.

Approximately 50% of the world's population are infected with *H. pylori*, infection usually occurring during childhood, and persisting throughout life, unless treated (Peek and Blaser 2002).

Chronic infection with *H. pylori* leads to inflammation which, depending on a range of host and environmental factors, can progress through a well-characterised process of atrophy, intestinal metaplasia, dysplasia, and cancer. Development of gastric cancer due to *H. pylori* infection has been linked to genetic mutations caused by chronic inflammation, an increase in epithelial proliferation, and loss of specialized glandular cell types such as parietal and chief cells (Fox and Wang 2001).

Several studies have shown that eradication of *H. pylori* from the stomach reduces the progression of atrophic gastritis and metaplasia, lowers the rate of gastric cancer recurrence, and prevents or delays the development of gastric adenocarcinoma (Uemura *et al.* 1997, Correa *et al.* 2000, Uemura *et al.* 2001).

H. pylori infection can also induce gastric cancer in rodent models, for example following infection over 1-2 years Mongolian gerbils have a higher rate of gastric adenocarcinoma (Watanabe *et al.* 1998, Honda *et al.* 1998).

1.4.1 Biological effects of *H. pylori* infection

H. pylori has a number of biological effects that contribute to the development of gastric cancer. One of the main effects on the gastric epithelium is inflammation. *H. pylori* activates the pro-inflammatory enzyme cyclooxygenase (COX)-2, which contributes to the production of inflammatory prostaglandins (Peek and Blaser

2002). It has been shown that co-culture of epithelial cells with *H. pylori* increases COX-2 expression in the gastric mucosa of infected individuals (Sawaoka *et al.* 1998, Fu *et al.* 1999). Expression of COX-2 is further increased in atrophic gastritis, metaplasia and gastric adenocarcinoma induced by *H. pylori* infection (Sung *et al.* 2000, Ristimaki *et al.* 1997).

H. pylori infection can also increase proliferation of epithelial cells. Co-culture of *H. pylori* with epithelial cells reduces the expression of cell cycle regulatory proteins such as p27, leading to arrest in G1 phase of the cell cycle (Shirin *et al.* 1999, Ahmed *et al.* 2000). Incubation of *H. pylori* with epithelial cells can also result in DNA damage through the synthesis of reactive oxygen species (Smoot *et al.* 2000). Proliferation can also be stimulated due to the host response to the *H. pylori* infection, hypergastrinaemia can result from infection, which stimulates proliferation of epithelial cells (see section 1.2.2.3).

H. pylori infection is associated with both increased and decreased levels of apoptosis in the gastric epithelium. *H. pylori*stimulates gastric epithelial cell apoptosis *in vitro* (Wagner *et al.* 1997, Fan *et al.* 1998, Peek *et al.* 1999), possibly by releasing the enzyme urease, which generates ammonia (Fan *et al.* 2000). Two studies on the effect of *H. pylori* on apoptosis showed that isolated gastric epithelial cells from infected patients had lower rates of apoptosis compared to uninfected samples (Peek *et al.* 1997, Rokkas *et al.* 1999).

Both increased and decreased rates of apoptosis could have detrimental effects. Increased rates of apoptosis could accelerate the progression to atrophic gastritis, and increase the risk of developing gastric cancer. However decreased apoptosis can lead

to a higher risk of mutation, predisposing to the development of cancer (Peek and Blaser 2002).

1.4.2 *H. pylori* infection and MMP/TIMP expression

Infection with *H. pylori* is known to increase the expression of MMPs, and this is significant in the remodelling process that occurs with infection, and may lead to gastric cancer (Wroblewski *et al.* 2003, Hemers *et al.* 2005, McCaig *et al.* 2006). The expression of MMP-1, -2, -7 and -9 has been shown to be induced in the gastric epithelium with *H. pylori* infection (Gooz *et al.* 2001, Wroblewski *et al.* 2003, Bebb *et al.* 2005, Bergin *et al.* 2004, Mori *et al.* 2003). *H. Pylori* induced increase in MMP-7 expression has a role in stimulating migration of gastric epithelial cells (Wroblewski *et al.* 2003).

Overexpression of TIMPs has been observed in gastric mucosal tissue with *H. pylori* infection (Gooz *et al.* 2001, Koyama 2004b, Bodger *et al.* 2008). However the literature relating to TIMP expression and *H. pylori* is limited.

The disruption in MMP and TIMP activity with *H. pylori* infection is likely to lead to breakdown of the extracellular matrix, increasing migration, also to the release of pro-inflammatory cytokines and chemokines, contributing to *H. pylori* induced inflammation (Sternlicht and Werb 2001).

1.5 Myofibroblasts

Myofibroblasts can be simply defined as smooth-muscle like fibroblasts. They show several distinguishing features e.g. prominent cytoplasmic actin microfilaments, adherens and gap junctions, and are contractile and motile. Immunohistochemical characterisation of myofibroblasts is usually by expression of α-smooth muscle actin, vimentin and desmin (Powell *et al.* 1999, Powell *et al.* 2005), although gastric myofibroblasts do not normally express desmin (Wu *et al.* 1999).

Myofibroblasts have important roles in a number of physiological processes. By secreting growth factors and their receptors, they are important in organogenesis and morphogenesis of tissues and organs. They also play a part in inflammation, wound healing, and turnover of the extracellular matrix (ECM) (Powell *et al.* 1999, Hinz *et al.* 2007).

Myofibroblasts are also involved in pathology in a variety of different tissue types. Increased proliferation of myofibroblasts is involved in fibrosis in many tissues including kidney, heart, liver, and lung, (Powell *et al.* 1999, Rocha *et al.* 2004, Willis *et al.* 2005, Poobalarahi *et al.* 2006, Díaz *et al.* 2007, Hinz *et al.* 2007). Stromal tissue, including myofibroblasts, can make up to 60-90% of cancer tissue, even though the origin of the cancer is epithelial. This gives a clear indication that mesenchymal-epithelial interactions have an important role in the development of cancer (Bhowmick *et al.* 2004, Gallagher *et al.* 2005, Powell *et al.* 2005, Sheehan *et al.* 2007).

Myofibroblasts are an important sub-epithelial cell type due to their ability to produce epithelial growth factors and their close proximity to the epithelial cells.

Previous work carried out in this laboratory has shown that *H. pylori* infection increases the production of MMP-7 by gastric epithelial cells (Wroblewski *et al.* 2003). MMP-7 acts as a myofibroblast growth factor by promoting the cleavage of IGFBP-5, secreted by myofibroblasts, to liberate IGF-II, which stimulates gastric epithelial cell proliferation and myofibroblast proliferation and migration. Over time increased numbers of myofibroblasts can lead to hyperproliferation and increase the chance of developing malignant transformation (Hemers *et al.* 2005, McCaig *et al.* 2006).

1.6 Epithelial-Mesenchymal signalling

Interactions between epithelial cells and mesenchymal cells are very important in maintaining normal mucosal organisation. This occurs during normal physiological processes such as development and wound healing, but also in pathology such as progression to cancer.

The microenvironment is thought to be of key importance in cancer progression. The extracellular matrix (ECM) is a complex structure that surrounds and supports the cells in all tissues of the body, and can regulate a number of cellular processes. The homeostasis of the microenvironment can be disrupted by epithelial-mesenchymal

interactions resulting in alteration in the architecture of the ECM. These changes can contribute to disease in many systems, including tumour progression and malignancy (Lukashev and Werb 1998, Brittan and Wright 2002).

Epithelial and sub-epithelial cells may release growth factors, cytokines and extracellular proteases, such as MMPs, and protease inhibitors, as a way to communicate with each other, and alter the microenvironment. Mutations that alter the structural properties of the ECM and the function of proteases, particularly the MMPs family often lead to disease, suggesting that ECM organisation is important for its function (Werb 1997).

An example of dysfunction in this system is *Helicobacter pylori* infection which causes inflammation of the gastric mucosa, and in some patients leads to gastric cancer (Correa & Chen 1994), and also prolonged hypergastrinaemia in patients with pernicious anaemia (PA) leading to ECL cell carcinoid tumours (Bordi *et al.* 1995). Progression to cancer in both cases involves epithelial remodelling with the loss and proliferation of certain cell types.

It has been shown that expression of a MMP-1 transgene in mouse epithelia caused an upregulation of endogenous MMP-1 in fibroblasts, resulting in neoplastic lesions and upregulation of other MMPs and ECM components (Thomasset *et al.* 1998). Also, results from our laboratory have shown that the release of MMP-7 from epithelial cells increases myofibroblasts numbers and stimulation of proliferation and migration, also MMP-7 can cause myofibroblasts to release IGF-II, which acts on myofibroblasts and epithelial cells, providing an example of epithelial-mesenchymal interactions altering the microenvironment (McCaig *et al.* 2006, Varro *et al.* 2007).

The altered expression of MMPs and TIMPs is likely to be an important mechanism in epithelial-mesenchymal signalling, and investigation of their contribution to the development of pathology is important.

1.7 MAPK signalling pathways

Mitogen activated protein kinases (MAPKs) are important enzymes that are involved in cellular regulation. The enzymes connect cell-surface receptors to intercellular regulatory targets. MAPK activity is regulated via three tiered phosphorylation cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MEK kinase (MAPKKK or MEKK) (English *et al.* 1999).

MAPKs have roles in most cellular processes e.g. embryogenesis, cell differentiation, cell proliferation and cell death. They are activated in the cytoplasm and translocate to the nucleus, directly or indirectly, through downstream kinases (Hazzalin and Mahadevan 2002).

In mammals there are four groups of MAPKs; the extracellular signal-related kinases (ERK)-1 and -2, Jun amino-terminal kinases (JNK)-1, -2 and -3, p38 proteins and ERK5. These are activated by specific MAPKs, ERK1/2 by MEK1/2, p38 by MKK3/6, JNKs by MKK4/7 and ERK5 by MEK5 (Chang and Karin 2001).

Several diverse cellular processes are controlled by MAPKs, in response to several different extracellular stimuli, and MAPKs have overlapping substrate specificities.

It is therefore important that MAPK activation is specific, and there are several mechanisms to ensure this. Scaffold proteins such as JIP1, which organises JNK1/2 and MKK7 into specific signalling cassettes, and MP1, that interacts with ERK1 and MEK1 to regulate ERK1 activation (Whitman *et al.* 1999, Schaeffer *et al.* 1998), are one mechanism of ensuring specificity. Specific MAPK activation also depends on sequential interactions between the members of a particular signalling cascade (Xia *et al.* 1998).

MAPKs are activated via a two-part enzyme-substrate reaction. The MAPKs recognise a phosphoacceptor site composed of serine or threonine followed by a proline. Specificity of this activation is ensured by a docking interaction with another site on the kinase that recognises a specific site on the substrate (Chang and Karin 2001).

1.7.1 ERK1/2 cascades

ERK1/2 are ubiquitously expressed proteins that can be stimulated by a wide variety of ligands and cellular stresses (Lewis *et al.* 1998), such as growth factors, cytokines, stresses and ligands of G protein-coupled receptors, and are responsible for mediating many cellular processes (Pearson *et al.* 2001). ERK1/2 are activated by MEK1/2 (Crews *et al.* 1992, Kosako *et al.* 1992). MEK1/2 are phosphorylated by Raf isoforms. The Raf family of protein kinases consists of A-Raf, B-Raf and c-Raf (Hagemann and Rapp 1999) (Fig 3).

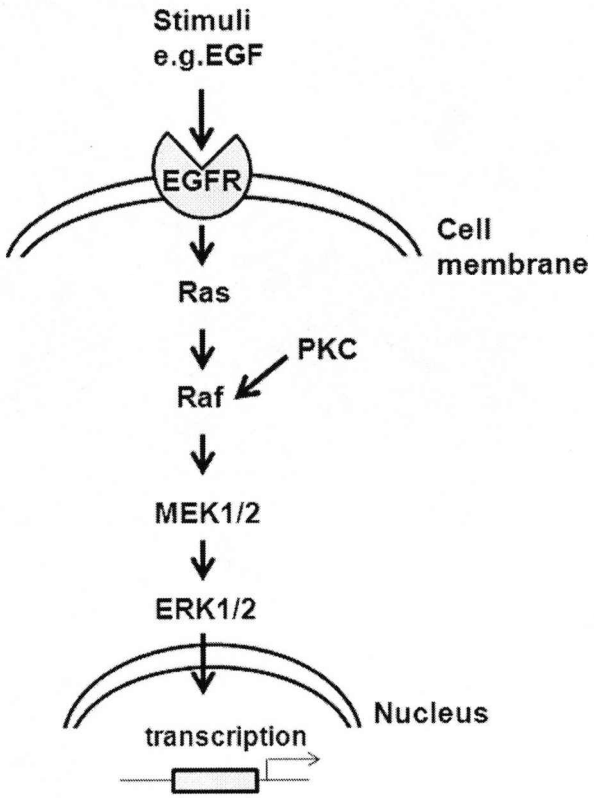


Fig 3: Overview of ERK1/2 signalling cascades. In response to stimulation by a variety of stimuli, Ras proteins stimulate Raf which phosphorylates MEK1/2. This activates ERK1/2 which influence gene transcription in the nucleus.

1.7.2 Functions of MAPK signalling

One of the best-studied functions of MAPK signalling cascades is regulation of gene expression in response to external stimuli (Treisman 1996).

Many MAPKs induce *fos* genes, Fos proteins then dimerise with Jun proteins forming the immediate early response transcription factor complex AP1, responsible for controlling the transcription of many genes (Treisman 1996), which regulate

many biological processes like cell proliferation and differentiation (Angel *et al.* 1991).

MAPKs function in the nucleus and target DNA-bound transcription factors, although they can also regulate gene expression via post-translational mechanisms involving targets in the cytoplasm. For example JNK stabilises IL-2 mRNA in activated T-cells (Chen *et al.* 2000).

MAPKs are involved in the stimulation of cell proliferation, for example by promoting cell-cycle progression (Palmer *et al.* 1998) and enhancing AP-1 activity (Treinies *et al.* 1999).

MAPKs can control cell survival via activation of ERK1/2, or induce apoptosis via JNK and p38 activation (Xia *et al.* 1995).

1.7.3 PKC signalling

Protein kinase C (PKC) is a signalling protein that forms part of a large family of multiple isoforms. These are involved in multiple biological processes. PKC can activate the ERK signalling cascade by stimulating Raf (Mellor and Parker 1998).

1.8 Growth factors

1.8.1 TGF-β

TGF-β is part of the TGF-β superfamily, which is composed of many cytokines including TGF-β, activins, inhibins, anti-müllerian hormone and myostatin.

There are three TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3, which are all highly similar.

TGF-β inhibits the proliferation of epithelial, endothelial and haematopoietic cells. However it can promote the proliferation of mesenchymal cells, and also induce the deposition of ECM, integrin expression, differentiation and migration. TGF-β also has a role in modulating the immune response (Letterio and Roberts 1998, Piek *et al.* 1999).

TGF-β signals via receptor serine/threonine kinases, known as type I and II receptors. This then activates members of the smad family of tumour supressors (Heldin *et al.* 1997).

The biological effects of TGF-β are context-dependent, i.e. they vary with tissue type and signalling by other factors/pathways.

TGF-β is expressed by all immune cell lineages, including B and T cells, it suppresses growth, differentiation and proliferation, which controls the immune system, preventing inappropriate autoimmune responses (Letterio and Roberts 1998).

TGF-β has a dual role in tumorigenesis. It is involved in inhibiting cell proliferation and therefore acts as a tumour suppressor in the early stages of cancer (Moustakas *et al.* 2002). However as cancer progresses cancer cells can develop resistance to the growth inhibitory effects of TGF-β, also cancer cells can release non-physiological levels of TGF-β which may affect tumour cell differentiation, and alter the microenvironment, thus contributing to tumour progression (Akhurst and Balmain 1999). Overexpression of TGF-β by tumour cells can also suppress the immune response which also allows tumour progression, as tumour cells escape immune attack (de Visser and Kast 1999).

There is evidence that TGF-β can induce epithelial-mesenchymal transition (EMT), via Ras/MAPK, PI3K signalling pathways, and via smad and RhoA activation. This contributes to cancer migration and invasion (Santibanez *et al.* 2000, Bhowmick *et al.* 2001).

1.8.2 PDGF

PDGF was discovered in whole blood serum in the 1970s (Ross *et al.* 1974), and originally purified from human platelets (Antoniades *et al.* 1979).

The PDGF family consists of four polypeptide chains, the "classical" chains, PDGF-AA and –BB, and the more recently discovered PDGF-CC and –DD chains (Li *et al.* 2000, Bergsten *et al.* 2001). The four different chains dimerise to form five different isoforms, PDGF-AA, -AB, -BB, -CC and –DD (Fredriksson *et al.* 2004).

The four PDGF genes have a broad pattern of expression, being expressed in most human tissues, although there are variations in the expression of the four genes. PDGF-D shows the lowest and most restricted expression (Fredriksson *et al.* 2004).

PDGF was originally discovered due to its ability to promote the proliferation of mesenchymal cells (Heldin *et al.* 1985), it is now known to have other functions such as promoting migration and cell survival (Claesson-Welsh 1994).

PDGF can activate several signalling systems including PI3K, PKC and Ras. The PDGF isoforms exert their effects by activating two different receptors, PDGFRα and PDGFRβ.

PDGF is crucial for development; in particular the development of many different mesenchymal cells depends on PDGFα, such as chondrocytes, lung alveolar smooth muscle and oligodendrocytes (Soriano 1997, Boström *et al.* 1996, Fruttiger 1999). It is thought that the main role of PDGFα in development is the promotion of proliferation of precursor populations. PDGFβ stimulation is essential for the development of vascular smooth muscle cells and pericytes (Lindahl *et al.* 1997).

In adult tissues PDGF has been shown to induce angiogenesis (Battegay *et al.* 1994, Nicosia *et al.* 1994), and to inhibit platelet aggregation (Bryckaert *et al.* 1989).

PDGF has an important role in wound healing, it acts on various cell types to aid healing. PDGF stimulates fibroblast and smooth muscle cell proliferation, and chemotaxis of neutrophils and macrophages. The production of other growth factors by macrophages is also stimulated (Heldin and Westermark 1999). PDGF can stimulate the production of matrix components such as fibronectin, collagen and proteoglycans (Blatti *et al.* 1988, Canalis 1981, Schönherr *et al.* 1991). Is also seems

that PDGF is important in remodelling following wound healing as it promotes the secretion of collagenase by fibroblasts (Bauer *et al.* 1985).

PDGF has a well known role in human disease, in particular many tumours express PDGF and its receptors, for example ovarian cancer, breast cancer, colorectal cancer, pancreatic cancer and gastric cancer (Apte *et al.* 2004, Carvalho *et al.* 2005, Sundberg *et al.* 1997, Ebert *et al.* 1995, Chung *et al.* 1992). PDGF overexpression is linked to tumour growth and progression, and is linked to poor prognosis. PDGF is also of importance in tumour angiogenesis.

PDGF has a role in paracrine stimulation of stromal cells. In many tumours it is found that tumour cells express PDGF while stromal cells express the PDGF receptors, showing that the tumour cells can promote stromal cell responsiveness to PDGF. This contributes to the formation of stroma-rich, highly vascularised tumours (Anan *et al.* 1996, Sundberg *et al.* 1997, Kawai *et al.* 1997).

PDGF expression, and PDGF receptor expression is increased in atherosclerosis, having involvement in the development of atherosclerotic lesions. A possible reason for this is low blood flow leading to increased PDGF production by endothelial cells (Mondy *et al.* 1997). The role of PDGF in these lesions may be to stimulate smooth muscle cells to migrate to the intima layer of the vessels and to proliferate and produce matrix molecules (Pompili *et al.* 1995).

Many human fibrotic conditions are linked to PDGF overexpression. Macrophages in the alveoli secrete PDGF in pulmonary fibrosis (Vignaud *et al.* 1991). This can cause proliferation of mesenchymal and epithelial cells in the lung, and the deposition of collagen (Yi *et al.* 1996).

PDGF overexpression is involved in kidney fibrosis, causing mesangial cell proliferation (Isaka *et al.* 1993). Other fibrotic conditions also involve PDGF, for example Dupuytren's contracture, rheumatoid arthritis and scleroderma (Alman *et al.* 1996, Pohlers *et al.* 2006, Zheng *et al.* 1998).

1.8.3 HGF

In the late 1980s two molecules, Scatter factor (SF), a potent motility factor (Stoker *et al.* 1987), and Hepatocyte growth factor (HGF), which was mitogenic for hepatocytes (Nakamura *et al.* 1989), were discovered independently. These were later found to be identical.

HGF is a large protein consisting of 6 domains, sharing similarities with plasminogen. HGF is synthesised as an inactive precursor (pro-HGF) which is converted by proteolysis into the active factor. Pro-HGF can be converted by several serine proteases, including plasminogen activator (uPA) (Birchmeier *et al.* 2003).

HGF is a multifunctional growth factor involved in cell proliferation, migration, differentiation and cell survival (Trusolino and Comoglio 2002).

HGF activates the oncogene c-Met. Met signalling induces proliferation and inhibits apoptosis in a variety of cell types. An example is the Madin-Darby canine kidney (MDCK) cell line which responds to HGF/Met signalling by "scattering", i.e. dispersal of cell colonies, epithelial-mesenchymal transition is induced, and mobility is increased (Stoker *et al.* 1987).

HGF is important in development and growth; it has essential roles in embryogenesis, such as liver development and skeletal muscle development in limb buds. There is also evidence that HGF is essential for placental development (Uehara *et al.* 1995, Bladt *et al.* 1995).

In adult tissues HGF is expressed in many tissues and is involved in physiological and pathological processes. HGF is upregulated after injury, suggesting a role in response to tissue damage (Nakamura *et al.* 2000, Rabkin *et al.* 2001). HGF also has an important role as a liver mitogen, enhancing liver regeneration (Michaelopoulos and De Frances 1997).

HGF signalling is also an important factor in cancer. HGF and/or Met are overexpressed by many types of cancer including thyroid, non small cell lung cancer, bone, renal and colorectal, overexpression is often linked to poor prognosis (Di Renzo *et al.* 1992, Olivero *et al.* 1996, Scotlandi *et al.* 1996, Natali *et al.* 1996 and Di Renzo *et al.* 1995).

HGF can signal via the ERK/MAPK cascade, affecting proliferation, cell-cycle progression, migration and invasion, and also altering the expression of extracellular matrix proteinases such as the MMPs. PI3K signalling can also be activated, which regulates cell survival (Birchmeier *et al.* 2003).

1.8.4 EGF

Epidermal growth factor (EGF) was originally discovered by Cohen in 1962, after it was found to stimulate precocious tooth eruption and eyelid opening in newborn mice.

EGF is a member of the EGF family of proteins all having very similar structural and functional characteristics. Other members of the family include heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor (TGF)-α, amphiregulin, epiregulin, epigen and betacellulin (Harris *et al.* 2003).

EGF acts by binding to epithelial growth factor receptor (EGFR). This receptor has an important role in influencing the behaviour of epithelial cells, and also tumours of epithelial origin. The EGFR consists of an extracellular receptor domain, a transmembrane region and an intracellular domain with tyrosine kinase function (Herbst 2004).

The EGFR is overexpressed in most human carcinomas (Salomon *et al.* 1995), and is responsible for autocrine and paracrine regulation of tumour progression. This overexpression has effects which include stimulation of proliferation, promotion of cell survival, increased invasion and metastasis (Normanno *et al.* 2005).

Activation of the EGFR is also important in angiogenesis, in healthy tissues and in cancer. EGF stimulation is thought to promote angiogenesis via stimulation of VEGF secretion, the main growth factor involved in angiogenesis. EGF stimulation promoted angiogenesis in this manner in glioma cells, gastric cancer cells and pancreatic cancer cells (Goldman *et al.* 1993, Maity *et al.* 2000, Akagi *et al.* 1003, Parikh *et al.* 2003).

Stimulation of the EGFR can exert its effects via Ras/PI3K and MAPK signalling pathways, which can then stimulate tumour cell migration, invasion and metastasis (Herbst *et al.* 2004).

The EGFR can be stimulated by gastrin, and has a role in gastric cancer progression (See section 1.2.5).

1.9 The TIMP Promoter

Control of TIMP expression involves factors such as regulation of gene expression, activation of zymogens and inhibition of active enzymes by specific inhibitors. Many MMPs and TIMPs are transcriptionally regulated by a variety of growth factors, cytokines and chemokines (Baker *et al.* 2002, Yan and Boyd, 2007).

It is likely that TIMP genes are downstream targets of immediate-early (IE) response genes such as the *Fos* and *Jun* genes that make up AP-1 (Clark *et al.* 2008).

TIMP-2 and TIMP-3 promoters contain multiple GC boxes in their proximal promoters which bind Sp1 and Sp3. However, these genes are still regulated by other mechanisms, such as NFκB, which has binding sites on TIMP-2 and -4 promoters, and Wnt signalling pathways (Clark *et al.* 2008).

The TIMP-1 promoter contains an AP-1 site with a neighbouring PEA3 site, and these are major mediators of transcription (Logan *et al.* 1996, Phillips *et al.* 1999).

Expression of the *TIMP-1* gene can be stimulated by PMA and transforming growth factor beta1 (TGF-β1). New protein synthesis is required for this induction, and the induction of AP-1 transcription factors closely precedes that of *TIMP-1*. Mutation or

deletion of the AP-1 site on the TIMP-1 promoter reduces the PMA induced response of TIMP-1 promoter-reporter constructs (Young *et al.* 2005).

Removal of an AP-1 site on the TIMP-1 promoter causes an almost complete loss of activity in liver hepatic stellate cells (HSCs), indicating that upregulation of TIMP-1 promoter activity may be at least partially controlled by an AP-1 dependent mechanism in these cells (Bahr *et al.* 1999).

Gardner *et al.* (2006) found that chronic downregulation of TIMP-1 promoter expression is regulated through multiple mechanisms, transcriptional control and loss of mRNA stabilization being the main contributors. This contributes to the progression of chronic neuroinflammation.

The *TIMP-2* gene promoter contains several Sp1 sequences, two AP-2 sites, three PEA3 sites and an AP-1 site (Hammani *et al.* 1996).

A polymorphism which abolishes the Sp1 site in the *TIMP-2* promoter is associated with COPD (Hirano *et al.* 2001) and breast cancer risk (Zhou *et al.* 2004).

The *TIMP-3* gene has a TATA box and single transcription start point. The proximal promoter has three GC boxes, and more distally contains six AP-1 consensus sequences, two NFκB sites and two p53 sites (Sun *et al.* 1995).

Murine *TIMP-3* regulation is linked to both AP-1 dependent and independent regulation. Mutational inactivation of an AP-1 site on the promoter abolished the activity of a TIMP-3 luciferase reporter construct (Kim *et al.* 1997).

Two different polymorphisms in the TIMP-3 promoter contribute to susceptibility to the chronic lung disorder pigeon breeders disease (Hill *et al.* 2004).

Amongst the *TIMP*s, *TIMP-4* displays tight tissue specificity (Leco *et al.* 1997). The TIMP-4 promoter contains an inverted CCAAT box and a Sp1 site. Mutation of the SP1 site abolishes reporter expression completely (Young *et al.* 2002).

The regulation of the *TIMP* genes is complex. They are regulated during development, in normal adult physiology and in disease. Control of gene expression at the promoter level is therefore very important, and a better understanding of this could offer an useful insight into a variety of biological processes.

1.10 Aims and Objectives

Objective 1:

Investigate regulation of TIMP expression in the gastric mucosa of humans and a mouse model of hypergastrinaemia

In all epithelial cancers, invasion of tumour cells through the basement membrane and local connective tissue is essential for metastasis, and MMPs have an important role in this process. TIMPs are the main specific inhibitors of MMP.

Previous work from our laboratory has shown increased expression of MMPs in the gastric mucosa of patients with elevated plasma gastrin concentrations and *H. pylori* infection (Wroblewski *et al.* 2002, Wroblewski *et al.* 2004).

Increased levels of TIMP-1, -2 and -4 have been found in gastric carcinoma biopsies (Koyama *et al.* 2004a, Kubben *et al.* 2006), and an association has been found between high TIMP levels and reduced survival in gastric cancer (Joo *et al.* 2000,

Yoshikawa *et al.* 2001). Previously published work from our laboratory has shown overexpression of TIMPs in gastric corpus biopsy tissue with *H. pylori* infection (Bodger *et al.* 2008).

The specific aims for this section will be:

- Investigate expression of TIMPs in transgenic mice that over express gastrin (Ins-GAS), and that are infected or not infected with *Helicobacter felis*
- Investigate expression of TIMPs in gastric biopsy samples from patients with elevated gastrin associated with pernicious anaemia (PA).

The method used to determine TIMP expression will be quantitative real time PCR (Q-PCR).

Hypothesis: Infection with *H. felis* will increase the expression of some or all of the TIMPs in the corpus of Ins-GAS mice.

Elevated plasma gastrin will cause increased TIMP expression in corpus biopsy samples from PA patients.

Objective 2:

Investigate the responsiveness of TIMP expression

Interactions between epithelial cells and mesenchymal cells are very important in maintaining normal mucosal organisation, however this also plays a significant part in pathology such as progression to cancer.

Myofibroblasts are an important sub-epithelial cell type due to their ability to produce epithelial growth factors and their close proximity to the epithelial cells.

Previous work carried out in this laboratory has shown that *H. pylori* infection increases the production of MMP-7 by gastric epithelial cells, which acts as a myofibroblast growth factor (Wroblewski *et al.* 2003). Increased numbers of myofibroblasts can lead to hyperproliferation and increase the chance of developing malignant transformations (Hemers *et al.* 2005, McCaig *et al.* 2006).

The role of MMPs in epithelial-mesenchymal signalling, and in the development of gastric cancer, makes investigating the role of their natural inhibitors, the TIMPs, in these processes relevant and important.

The specific aims for this section will be:

- Investigate the cellular expression of TIMPs in the human gastric mucosa
- Investigate the expression and localisation of TIMPs in a gastric cancer cell line, AGS-G_R
- Investigate the response of TIMP expression to growth factors and gastrin
- Investigate the biological relevance of TIMPs through functional assays e.g. migration or invasion

The main methods used in this section will be immunohistochemistry, Immunocytochemistry, Western blotting and migration assays.

Hypothesis: TIMP expression will be influenced by growth factors and gastrin stimulation. TIMPs will have an effect on cellular processes such as migration.

Objective 3:

Investigate the cellular mechanism and responsiveness to stimulation of TIMP expression

The expression of TIMPs in response to various conditions such as *Helicobacter* infection, elevated plasma gastrin levels and growth factor stimulation will have been investigated.

Dissection of the mechanism of stimulation of TIMP expression is relevant in order to give greater understanding into the role of TIMPs in the organisation of the gastric epithelium, and biological processes in normal and pathological conditions.

The specific aims of this section will be:

- Investigate the regulation of TIMP luciferase expression in myofibroblasts and/or AGS-G_R cells, in response to growth factor or gastrin stimulation
- Dissect signalling pathways for TIMP expression

The main methods used to achieve this will be luciferase-reporter assays in myofibroblasts or AGS-G_R cells transfected with TIMP-luc reporter constructs.

Chapter 2

Materials & Methods

2.1 Materials

Cell culture media was obtained from Sigma (Poole, UK), FBS from Hyclone (Cramlington, UK), antibiotic-antimycotic, penicillin/streptomycin and non-essential amino acid solution from Sigma. Cell culture plastic ware was obtained from Invitrogen (Paisley, UK). Trypsin and EDTA were obtained from Sigma.

TRI-Reagent was supplied by Sigma. DNAse and DNAse buffer, random hexamers, AMV-reverse transcriptase, AMV buffer and RNAsin were obtained from Promega (Southampton, UK). Restriction enzymes were obtained from Sigma or New England Biolabs (Herts, UK), restriction enzyme buffers were also from Sigma or NEB and BSA was obtained from Promega. DNA hyperladder 1 was from Bioline (London, UK). T4 DNA ligase and T4 buffer were from Promega. Klenow enzyme was bought from Bioline. Competent cells were obtained from Bioline.

Protein ladder was obtained from Fermentas Life Sciences (York, UK); nitrocellulose membrane was from Amersham Biosciences (Bucks, UK). Primary antibodies were obtained from RDI (Cambridge, UK), Chemicon (Herts, UK) or Santa-Cruz (Wembley, UK). GAPDH antibody used for Western blotting was from Biodesign (Abingdon, UK). Western Blot secondary antibodies were obtained from Sigma or Pierce (Northumberland, UK), Immunocytochemistry secondary antibodies were from Jackson ImmunoResearch (Suffolk, UK). Chemiluminescence substrate was obtained from Pierce and film was obtained from Amersham Biosciences. Protease inhibitor and phosphatase inhibitor were bought from Calbiochem,

(Nottingham, UK) and RIPA buffer from Upstate (Herts, UK). Vectashield was obtained from Vector (Peterborough, UK).

Nucleofection solutions were obtained from Amaxa (Koln, Germany). Combi-mag was obtained from Oz Biosciences (Marseilles, France), TransFast transfection reagent was from Promega.

Renilla (ph-SV40) and pGL4 luciferase vectors were bought from Promega, and luciferase reporter assay kits were from Promega.

Inhibitor RO-320432 was obtained from Alexis Biochemicals, UO-126 was obtained from Cell Signaling Technology (Herts, UK) and LY-294002 was obtained from Promega.

Q-PCR primers and probes, 18s kit and master-mix were bought from Eurogentec (Southampton UK).

All other general chemicals and reagents were obtained from Sigma.

2.2 Cell Culture

2.2.1 Human gastric myofibroblasts

Myofibroblasts were prepared from human gastric corpus using a published method (Wu *et al.* 1999). Myofibroblasts were maintained in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% foetal bovine serum (Hyclone,), 2% antibiotic-antimycotic (Sigma), 1% penicillin/streptomycin (Sigma) and 1% non-

essential amino acid solution (Sigma). Cells were maintained at 37°C. Medium was replaced every 48-60 hours. Myofibroblast identity was confirmed by positive staining for α-SMA and vimentin.

All work involving primary human tissue was approved by the local Ethics Committees; all patients gave informed written consent.

2.2.2 AGS-G_R cells

AGS-G_R cells are a human gastric cancer cell line stably transfected with a vector encoding the gastrin-CCK2 receptor (CCK2R) as described by Watson *et al.* (2001). AGS-G_R cells were cultured in nutrient mixture F-12 HAM (Sigma) supplemented with 10% foetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Sigma). Cells were maintained at 37°C. Medium was replaced every 2-3 days.

2.3 RNA extraction

2.3.1 Tissue

50-100mg of mouse tissue was homogenised in 1ml TRI Reagent (Sigma). The homogenised lysate was centrifuged at 12,000g for 10 min at 4°C to remove insoluble material. The clear supernatant was transferred to a clean tube, allowed to stand at room temperature for 5 min before adding 0.2ml chloroform. The sample was shaken vigorously by hand for 15s, allowed to stand for 5 min at room

temperature, and centrifuged at 12,000g for 15 min at 4°C. After centrifugation the mixture separated into 3 phases, the colourless upper aqueous phase containing RNA was transferred to clean 1.5ml tubes, and 0.5ml isopropanol added. The sample was mixed and allowed to stand for 5-10 min at room temperature before centrifuging at 12,000g for 10 min at 4°C. Centrifugation results in a precipitated RNA pellet. The supernatant was removed and the RNA pellet washed in 1ml ice cold 70% ethanol, vortexed and centrifuged at 7500g for 5 min at 4°C. The pellet was then air-dried for 5-10 min and re-dissolved in an appropriate volume of DEPC treated water. The RNA concentration and purity was estimated spectrophometrically at 260 and 280nm.

If the RNA was to be stored, following washing the RNA pellet with 70% ethanol, 1ml ice cold absolute ethanol and 20µl 3M sodium acetate (in DEPC treated water) were added, and this was stored at -80°C.

2.3.2 Adherent cells

Cells were grown until confluent in a $10cm^2$ culture dish, the medium discarded and 1ml TRI Reagent (Sigma) added directly onto the dish to lyse the cells. The lysate was passed through a pipette until homogeneous. RNA was then extracted from the homogenised lysate as described above. Following extraction the RNA was precipitated and stored under ethanol at -80°C.

2.4 Molecular Biology

2.4.1 DNAse treating of RNA samples

In order to precipitate RNA, pellets stored in sodium acetate/ethanol were microfuged at max speed for 15min at 4°C, the supernatant was carefully discarded and the pellet briefly air dried. The dried pellet was washed in 1ml ice cold 70% ethanol, and again in ice cold absolute ethanol. After the final wash the pellet was air dried for 10-15 min before resuspending in 20-30µl DEPC water, depending on size of pellet, and gently shaking for 15-30 min.

The RNA concentration and purity was estimated spectrophotometrically at 260 and 280nm.

30µg (less if amount of sample was limiting) RNA was treated with 30µl (1unit/µg RNA) DNAse (Promega), and 5µl 10x DNAse buffer, made up to a total reaction volume of 50µl with DEPC treated water. This mixture was incubated at 37°C for 30min. The reaction volume was increased to 200µl with DEPC-treated water and an equal volume of acid phenol chloroform added. The sample was vigorously mixed and centrifuged at max speed for 5 min. The top aqueous layer, containing the RNA, was removed and to this 1ml ice cold absolute ethanol and 20µl 3M sodium acetate (in DEPC treated water) was added. The sample was stored at -80°C and re-precipitated when required.

2.4.2 Reverse transcription of RNA

RNA stored under ethanol at -80°C was precipitated as described above and resuspended in 20-30µl DEPC water, according to pellet size. The RNA concentration and purity was estimated spectrophotometrically at 260 and 280nm.

In most cases 5µg of RNA was reverse transcribed, however if the amount of RNA present was inadequate less was used (1µg minimum). The required volume of RNA (maximum 18.5µl) was pipetted into sterile tubes, and 0.5µg random hexamers (Promega) added and the volume increased to 10µl with DEPC treated water if necessary. In order to anneal the primer and RNA the sample was heated to 70°C for 5min, and allowed to cool slowly to less than 40°C by switching off the heat block and leaving the tubes in place. After cooling the following reagents were added to the sample in the order stated; 6µl 5xAMV buffer (Promega), 1mM dNTP mix (stock 12.5mM), 20u RNAsin (Promega), and 15u AMV-RT (Promega). The reaction volume was increased to 30µl with sterile distilled water. The reaction mixture was left to proceed at room temperature for 10min before incubation at 42°C for one hour. The reaction was stopped by heating to 95°C for 5 min, and the cDNA was stored at -20°C. Any unused RNA was re-precipitated as described above and stored at -80°C.

2.4.3 Restriction Enzyme Digests

In each restriction digest 1-2µg purified plasmid DNA was used, with 1 unit enzyme per µg DNA. The final reaction volume was 25µl for single enzyme reactions, or 30µl for double digests. The appropriate 1x buffer was used, buffers were supplied at

a 10x concentration so 2.5µl was added per 25µl digest. When required, BSA (Promega) was added at a final concentration of 100-200µg/ml. The reaction was made up to the final volume with sterile distilled water.

The digest was allowed to proceed at the optimum temperature for the enzymes used for 1.5-2 hours. See appendix 1 (Chapter 8.1) for enzymes used and reaction conditions.

2.5 Plasmid Preparation

2.5.1 Elution of DNA from agarose gel

The DNA sample (≤5µg) was separated on a standard 0.8% agarose gel in TBE (Tris-borate/EDTA) buffer. The DNA fragment was visualised with UV light and excised from the agarose gel with a clean scalpel. A QIAGEN MinElute Gel Extraction Kit (for extraction of DNA fragments 70bp – 4Kb) was used to extract the DNA (Qiagen, Crawley UK).

The gel slice was weighed in a 1.5ml tube, and 3 volumes of Buffer QG (see chapter 8.2 for compositions) were added to 1 volume of gel (100mg ~ 100µl). The maximum weight of gel slice added per spin column was 400mg. The gel slice and buffer mixture was incubated at 50°C for 10 min, with vortexing every 2–3 min. After the gel slice had dissolved completely 1 gel volume of isopropanol was added and the sample was mixed by inverting the tube several times. A Qiagen MinElute column was placed in a 2ml collection tube, the sample was applied to the column

and centrifuged for 1 min at 10,000g, in order to bind the DNA. The flow-through was discarded and 500µl of Buffer QG was added to the spin column which was centrifuged for 1 min at 10,000g. The flow-through was discarded and 750µl of Buffer PE was added to the column to wash the DNA, this was centrifuged for 1 min at 10,000g. The flow-through was discarded and the column centrifuged again for an additional 1 min at 10,000g. The column was placed into a clean 1.5ml microcentrifuge tube and to elute the DNA 10µl of Buffer EB (10mM Tris-Cl, pH 8.5) was pipetted into the centre of the membrane, the column left to stand for 1 min, and then centrifuged for 1 min at 10,000g.

2.5.2 DNA Ligation

Each ligation reaction included 50ng vector DNA with a 3:1 molar ratio of insert:vector DNA. Amounts of vector and insert were estimated by running on an agarose gel and comparing to 5µl DNA hyperladder 1 (Bioline).

To the appropriate volume of insert and vector DNA, 1µl T4 DNA Ligase (Promega) and 1µl T4 10x Buffer (Promega) were added in a total reaction volume of approximately 10µl (made up with sterile distilled water if necessary). This reaction was left to proceed at 4°C for 16-72 hours.

2.5.3 Transformation of competent cells with plasmid DNA

For transformation with purified plasmid DNA, the competent cells used were α-Select Chemically Competent Cells – Silver efficiency (Bioline). For

transformations using ligations α-Select Chemically Competent Cells – Gold efficiency (Bioline) were used. The cells were stored at -80°C and prior to transformation were thawed on wet ice. After mixing gently 50µl competent cells were added to 2µl ligation or 1µl plasmid DNA and this mixture was left on ice for 20 minutes. The cells were then heat-shocked at 42°C for 45s then returned to ice for 2 minutes. The transformation reaction was diluted by adding 1ml SOC (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 20mM glucose) and the tubes were shaken at 225rpm for 1 hour at 37°C. 200µl of the transformed cells were plated onto LB agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.2% agar) plates containing appropriate selective antibiotic and incubated overnight at 37°C.

2.5.4 Purification of plasmid DNA

2.5.4.1 Purification of up to 500µg plasmid DNA

After transforming competent cells and growing on LB agar plates, a single colony was picked from the plate and a starter culture of 3ml LB medium containing the appropriate selective antibiotic was inoculated. This was incubated for 6-8 h at 37°C with shaking at 225 rpm.

1ml of the 3ml starter culture was diluted in 100ml LB medium containing the appropriate selective antibiotic. This was grown at 37°C overnight with shaking at 225rpm. The bacterial cells were harvested by centrifugation at 6000g for 15 min at 4°C. A QIAGEN plasmid maxi kit was used to purify the DNA. The bacterial pellet was resuspended in 10ml Buffer P1 (with RNaseA added to give a final

concentration of 100µg/ml), see Chapter 8.2 for buffer compositions. 10ml Buffer P2 was added and the mixture was incubated at room temperature for 5 min. 10ml of chilled Buffer P3 was added and the mixture was mixed and incubated on ice for 20 min. The mixture was centrifuged at 20,000g for 30 min at 4°C, the supernatant containing plasmid DNA was centrifuged again at 20,000g for 15 min at 4°C. While centrifuging a QIAGEN-tip 500 was equilibriated by applying 10ml Buffer QBT. The supernatant containing the plasmid DNA was applied to the QIAGEN-tip and after this had flowed through the tip was washed with 2 x 30ml Buffer QC. The plasmid DNA was eluted with 15ml Buffer QF and the DNA precipitated by adding 10.5ml room-temperature isopropanol. This was centrifuged immediately at 15,000g for 30 min at 4°C. The supernatant was discarded and the DNA pellet washed with 5ml of room-temperature 70% ethanol, and centrifuged again at 15,000g for 10 min. The supernatant was carefully removed and the pellet dried in a flow hood for 5–10 min. The DNA pellet was redissolved in 500µl 10mM Tris-Cl pH 8.5. The yield was determined and purity estimated by spectrophotometry at 260 and 280 nm.

2.5.4.2 Purification of up to 20µg plasmid DNA

After transforming competent cells and growing on LB agar plates a single colony was picked from the plate and a starter culture of 3ml LB medium containing the appropriate selective antibiotic was inoculated. This was incubated overnight at 37°C with shaking at 225 rpm. A Sigma GenElute™ Plasmid Miniprep Kit was used to purify the plasmid DNA. The cells were pelleted by centrifugation at 12,000g for 1 min and resuspended in 200µl resuspension solution (with RNase A added to give a final concentration of 100µg/ml), see Chapter 8.2 for buffer compositions. After

mixing, 200µl of lysis solution was added and the mixture incubated at room temperature for up to 5 min before adding 350µl of neutralization solution and inverting 4-6 times to mix. The mixture was then centrifuged at max speed for 10 min. The cleared lysate was transferred into a Mini Spin Column in a collection tube and centrifuged at 12,000g for 1 min. The flow-through was discarded and 750µl wash solution added to the column. This was centrifuged at 12,000g for 1 min, the flow through was discarded and the column centrifuged again at 12,000g for 2 min to dry the column. The column was transferred to a new collection tube and 100µl elution solution added. This was centrifuged at 12,000g for 1 min to elute the DNA. The yield was determined by spectrophotometry at 260 nm.

2.6 Western Blotting

2.6.1 Protein extraction from whole cells

The medium was removed from the cells and reserved for concentration (see below). 10µl each protease inhibitor cocktail set III and phosphatase inhibitor cocktail set II (Calbiochem) were added to 1ml RIPA buffer (Upstate). The cells were washed once with sterile 1xPBS, 2ml 0.02% EDTA solution (Sigma) was then added, to detach the cells. 8ml full medium was then added to the cells followed by centrifugation at 800g for 7min. The cell pellet was resuspended in 80µl RIPA buffer, sonicated for 10min and left on ice for 30min. Cells were then centrifuged at 20,000g for 7 min at 4°C. The supernatant, containing protein, was stored at -80°C.

2.6.2 Concentrating conditioned medium

Medium was removed from the cells and centrifuged at 800g for 7min, to pellet any remaining cells or debris. The supernatant was placed into the reservoir of a medium concentrating column (Millipore, Watford UK) and centrifuged at 2000g for 20min. Flow-through was discarded and remaining medium added to the column. This was repeated until 1.5ml medium remained in the column reservoir. The medium was placed into a 15ml tube and whilst vortexing, an equal volume of ice cold 20% TCA was slowly added. The mixture was left on ice for 2h then centrifuged at 20,000g at 4°C for 10min. The protein pellet was washed twice with cold 100% ethanol and resuspended in a suitable volume (50-100µl) RIPA buffer, depending on the size of the pellet, and stored at -80°C.

2.6.3 Protein estimation

Protein estimation was carried out using a Bio-Rad DC protein assay kit (Bio-Rad, Herts UK). Standards of 0, 0.2, 0.5, 0.75, 1 and 1.44 mg/ml BSA, made up in RIPA buffer, were used to generate a standard curve. All standards and samples were measured in triplicate; samples were diluted 1:5 in RIPA buffer. The assay was carried out in a flat-bottomed 96-well plate. 5µl standard or diluted sample were pipetted into triplicate wells. 1ml solution A was mixed with 25µl solution S, and 20µl dispensed to each well containing sample or standard. 200µl solution B was then added to each sample and the plate was left at room temperature for 15 min. If the samples appeared darker than the standards by eye, the assay was repeated using a 1:10 sample dilution. The amount of protein was measured by reading each sample

at 750nm using a Packard SpectraCount plate reader (Packard, UK). A standard curve was drawn manually using the measurements from the standards, and this was used to calculate the amount of protein present in the samples.

2.6.4 Western Blotting

Protein lysates and media samples were separated on 12% SDS-polyacrylamide gels, made up using the quantities of reagents shown in table 3:

Resolving Gel	**12%**
Acrylamide/Bis	9.9ml
ddH_2O	8.375ml
1.5M Tris-HCl pH 8.8	6.25ml
10% (w/v) SDS	0.25ml
10% ammonium persulphate (fresh)	0.125ml
TEMED	0.012ml

Table 1: quantities of reagents used to make SDS-polyacrylamide gels. These quantities make 4 gels using 1.5mm plates.

The gels were set for 30-40 min at room temperature; a 4% stacking gel was then poured (1.3ml Acrylamide/Bis, 6.1ml ddH_2O, 2.5ml 0.5M Tris-HCl pH 6.8, 0.1ml 10% (w/v) SDS, 0.05ml 10% ammonium persulphate (fresh) and 0.01ml TEMED). Combs were inserted and the gel set for 30-40 min at room temperature.

The protein and media samples to be loaded were prepared by adding the appropriate volume of 6x Laemli buffer (125mM Tris-Cl pH 6.7, 6% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.1% bromophenol blue). Before loading onto the gel each sample was heated to 99°C for 5 minutes. 5µl PageRuler™ Prestained Protein Ladder (Fermentas Life Sciences) was loaded in one lane on each gel. The proteins were electrophoresed at 75V for 1.5-2 hours, until the dye migration front reached the bottom of the gel, in 400ml 1x running buffer per gel tank (5x running buffer pH 8.3; 15g Tris base 25mM, 72g Glycine 192mM, 5g 0.1% SDS in 1L ddH_2O).

The proteins were then transferred onto nitrocellulose membranes (Amersham Biosciences), at 100V for 1 hour exactly. Tanks were filled with transfer buffer (11.3g Glycine, 2.4g Tris Base, 200ml Methanol in 1L ddH_2O). After transfer the membranes were blocked in 5% non-fat milk in TBS-tween (0.1%). Primary antibodies were added in 5ml blocking solution and membranes were incubated in primary antibody overnight on a rocking table at 4°C (see chapter 8.4 for details). Primary antibodies were followed by HRP-conjugated secondary antibodies (Pierce) all diluted in blocking buffer (see chapter 8.4 for dilutions). Incubation with secondary antibody took place on a rocking table for 1 hour at room temperature. Before and after addition of the secondary antibody the membrane was washed by adding 3x10ml TBS-tween (0.1%) for 10 min per wash. Detection was by

SuperSignal West Pico Chemiluminescence substrate (Pierce) and Hyper Film (Amersham Biosciences).

Blots were re-probed with GAPDH (Biodesign) as a control for equal loading. Before adding the GAPDH antibody the membranes were washed 3x 10 min in TBS-tween (0.1%) then blocked for 1-2h in 5% non-fat milk in TBS-tween (0.1%). The GAPDH antibody was used at a 1:10,000 dilution, probed overnight at 4°C with shaking. The secondary antibody used was rabbit anti-mouse (Pierce) 1:5000 dilution in blocking buffer, this incubation took place for 1h at room temperature with shaking.

2.8 Immunocytochemistry

Cells were seeded directly into 4-well chamber slides, in full serum medium (FSM) and left for at least 24h. Cells were then serum starved for 24h, washed twice in PBS and fixed by incubating in 500µl 4% paraformaldehyde per well for 30 min at room temperature. Cells were washed twice in 1ml PBS then either stored under PBS at 4°C for up to 1 week, or used for immunocytochemistry immediately. To carry out immunocytochemistry cells were incubated in 500µl PBT (0.3% BSA, 0.2% triton-X in PBS) for 30 min at room temperature. After incubation cells were washed twice in PBS and incubated in 500µl 5% BSA for 10 min at room temperature. Cells were washed twice in PBS and incubated in 10% donkey serum for 30 min at room temperature. Cells were washed twice again in PBS before adding the primary antibody, 200µl per well diluted in PBS in a moist environment,

and incubated overnight at 4°C (see chapter 8.5 for primary antibodies used). After the primary antibody incubation the cells were incubated for 10 min at room temperature in 0.14M NaCl (in PBS) followed by an incubation in 0.5M NaCl (in PBS) for 10 min at room temperature, and a further incubation for 10 min in 0.14M NaCl (in PBS). The FITC-conjugated secondary antibodies (Jackson ImmunoResearch) were then added, made up in 10mM HEPES pH7.6 1:400 dilution, and incubated at room temperature for 1 hour in the dark. Cells were then washed 3 times in PBS then mounted in Vectashield containing DAPI (Vector) to counter stain nuclei.

Slides were examined using a fluorescence microscope and images processed using AxioVision 4.6 imaging software (Zeiss, Welwyn Garden City, UK). Ten fields of three replicates were counted and results expressed as percentages of total cell number.

2.9 Transfection

2.9.1 Myofibroblasts

2.9.1.1 Nucleofection

Cells were grown to 80-100% confluency in T75 culture flasks, and 2ml trypsin added per flask to detach cells. 8ml serum free medium (SFM) was added to the detached cells which were counted using a haemocytometer. 0.5-1x10^6 cells were used per nucleofection sample; these were centrifuged at 800g for 7 minutes and

resuspended in 120µl Human AoSMC nucleofector™ solution (Amaxa) per sample required.

1-5µg highly purified plasmid DNA and 10µg renilla (phRL-SV40, Promega) reporter construct were used per transfection sample, 100µl cell solution was added to the DNA. 120µl of this transfection mixture was pipetted into an amaxa cuvette and each sample was then nucleofected using amaxa Nucleofector™ machine program U-25. Each transfection was carried out in triplicate.

After transfection 500µl of FSM was added to the cuvette and this was transferred to a clean tube. The transfected cells were transferred to a 6-well plate, an equal amount of cells being added per well. The transfected cells were left at 37°C for 48h.

2.9.1.2 Magnetofection

Cells were grown to 80-100% confluency in T75 culture flasks. To detach cells 2ml trypsin was added per flask. 8ml SFM was added to detached cells which were counted using a haemocytometer. 100,000 cells were seeded per well of a 6-well plate, each well containing 2ml FSM. The plate was left at 37°C for approximately 24h.

Cells were transfected in 0.8ml SFM per well. The transfection mixture was made up as a master mix, so 0.8ml per well SFM was added to a sterile tube. To this 20ng/well Renilla and 1µg/well of the required plasmid DNA was added. The mixture was then vortexed for 20-30s before adding 1µl/µg DNA of CombiMag reagent (Oz Biosciences). After vortexing for 20-30s 6µl/µg TransFast™ transfection reagent (Promega) was added, then the mixture vortexed again for 20-

30s. The mixture was then incubated for 20min at room temperature. After washing each well twice with serum free medium 0.8ml of the transfection mixture was added per well and plates were incubated on magnetic plates (Oz Biosciences) for 15min at room temperature. After this incubation 1.5ml FSM was added to each well and the plates were returned to a 37°C incubator, and left for approximately 48h before stimulation/harvesting. Each transfection was carried out in triplicate.

2.9.2 AGS-G_R cells

2.9.2.1 Nucleofection

Cells were grown in T75 culture flasks, and detached by adding 2ml trypsin per flask. 8ml SFM was added to the detached cells which were counted using a haemocytometer. The cells were then centrifuged at 800g for 7 minutes, washed with PBS and then centrifuged again at 800g for 7 minutes. The liquid was then drained from the cell pellet, which was re-suspended in 120µl cell line nucleofector solution (Amaxa) per sample required. The re-suspended cells were added to 1.5ml tubes containing the DNA/siRNA to be transfected. This was mixed by pipetting up and down. 100µl of this solution was placed in a cuvette and nucleofected using amaxa Nucleofector™ machine program U-25. 500µl full medium was added to the nucleofected cells, which were transferred to a clean tube. The transfected cells were then placed in T25 tissue culture flasks, 10cm^2 culture dishes or chamber slides as required.

2.9.2.2 Lipofection

Cells were grown to 80-100% confluency in T75 culture flasks. Cells were counted and 250,000 cells were seeded per well of a 6-well plate, each well containing 2ml full medium. The plate was left at 37°C for approximately 24h.

Cells were transfected in 0.8ml SFM per well. The transfection mixture was made up as a master mix, so 0.8ml per well SFM was added to a sterile tube. To the SFM 1.5ng/well Renilla and 0.2-1μg/well of the required plasmid DNA was added. The mixture was then vortexed for 20-30s before adding 6μl/μg TransFast™ transfection reagent (Promega), the mixture was then vortexed for 20-30s and incubated for 20min at room temperature. After washing each well twice with serum free medium 0.8ml of the transfection mixture was added per well and plates were incubated at 37°C for 1 hour. After this incubation 2ml FSM was added to each well and the plates were returned to a 37°C incubator, and left for approximately 24h before stimulation/harvesting. Each transfection was carried out in triplicate.

2.10 Luciferase Reporter Assays

Transfected cells were stimulated in 2ml SFM after washing each well twice with SFM. After the last wash all traces of medium were removed with a sterile pipette. Stimulation took place at 37°C for 4-24h, depending on individual protocols.

To harvest cells after stimulation, each well was washed twice with non-sterile PBS and 0.5ml 1x Passive Lysis Buffer (Promega) was added. The plates were then either read immediately or stored at -80°C for up to 48h.

Luciferase assays were performed using the dual-luciferase reporter assay system (Promega), in a Lumat LB9507 (E G & G Berthold) luminometer. The luminometer was programmed to inject 100µl of each reagent per sample, and to provide a 3s delay and 10s measuring period per sample. 20µl from each well was transferred to a plastic tube for measurement. Each well was read once.

Chapter 3

Expression and regulation of TIMPs in human and murine stomach by *Helicobacter* and gastrin

3.1 Introduction

The breakdown of the extracellular matrix (ECM) is an essential part of many normal processes in the stomach, such as development and tissue remodelling after injury. It is also important in pathology such as gastric ulcer, chronic inflammation and cancer, where processes such as local invasion, angiogenesis and vascular invasion all contribute to disease progression (Nagase and Woessner 1999, Sternlicht and Werb 2001).

As I mentioned in the introduction, the proteinases mainly responsible for the breakdown of the ECM are the matrix metalloproteinases (MMPs).

Increased expression of MMP-9 enhances invasion of the ECM by the human gastric cancer cell line, AGS. Also, increased expression of MMP-9 was found in the gastric mucosa and ECL carcinoid tumours of patients with elevated gastrin concentrations (Wroblewski *et al.* 2002). It is known that *H. pylori* infection in the stomach predisposes some patients to gastric cancer (Uemura *et al.* 2001), and MMP-7 may have a role in the response of gastric epithelial cells to *H. pylori* infection and progression to cancer. Indeed *H. pylori* infection is associated with increased MMP-7 expression in gastric epithelial cells, and this has a role in stimulating their migration (Wroblewski *et al.* 2004).

In gastric cancers like any other epithelial cancers, in order for tumours to become invasive and metastasise they must be able to break through the basement membrane, and invade through local connective tissue to metastasise to distant organs (Kleiner 1999). MMPs are important in this process.

MMPs are regulated in several ways; the main specific inhibitors of MMP activity are the tissue inhibitors of matrix metalloproteinases (TIMPs).

Increased levels of TIMP-1, -2 and -4 have been found in gastric carcinoma biopsies (Koyama *et al.* 2004a, Kubben *et al.* 2006), and an association has been found between high TIMP levels and reduced survival in gastric cancer (Joo *et al.* 2000, Yoshikawa *et al.* 2001). Some TIMPs have roles in activating MMPs, for example a MMP-2/TIMP-2/MT1-MMP complex is formed on the cell surface, and releases MMP-2 when cleaved. This could be a factor in the increased TIMP expression seen in gastric cancer. Moreover, the TIMPs can affect cell growth and survival of the gastric cancer cells independently of their roles in MMP activation and inhibition (Koyama *et al.* 2004a). In a study by Bergin *et al.* (2004), there was no difference in TIMP-1 or -2 expression with *H. pylori* status; however infected individuals had higher MMP-9 activity. This imbalance in MMP and TIMP activity could have an important role in *H. pylori* induced gastritis, allowing MMP-induced degradation of the ECM.

Conversely, however, Western blot data from our laboratory show an increase in TIMP -1, -3 and -4 expression with *H. pylori* infection in the human gastric corpus. There was no change in the expression of TIMP-2 (fig 1).

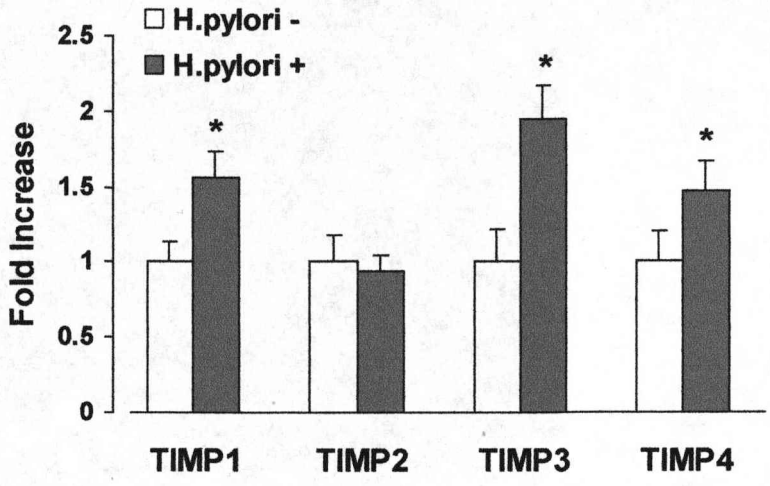


Fig 1: Expression of TIMP-1, 3 and 4 increased in *H. pylori* infected human gastric corpus. Results from Western blot quantified by densitometry and normalised to GAPDH. (*H.pylori* negative n=13, *H.pylori* positive n=12). Data shown as fold increase ± SE *p<0.05 Student's t-test. (Data supplied by A. Varro).

The aim of this part of the work was to investigate changes in TIMP expression in the gastric mucosa of human and a murine model. Quantitative real time PCR (Q-PCR) was used to investigate expression of TIMPs in transgenic mice that over express gastrin (Ins-GAS), and that are infected or not infected with *Helicobacter felis*, and also in gastric biopsy samples from patients with elevated gastrin associated with pernicious anaemia (PA).

3.2 Materials and Methods

3.2.1 Samples

Samples of full thickness gastric corpus were taken from Ins-GAS mice. The Ins-GAS mice contain a transgene in which a rat insulin promoter drives a human gastrin minigene. Amidated gastrin is produced in β cells of the pancreas, and the mice have elevated gastrin levels. The mice were infected with *H. felis* and samples taken for RNA extraction at 3 and 6 months after infection, as previously described (McCaig *et al.* 2006).

Endoscopic pinch biopsies of gastric corpus were obtained from patients undergoing routine gastroscopy for investigation of dyspepsia. Samples were selected on the basis of plasma gastrin concentration. *H. pylori* status was assessed by serology, antral urease test and histology. Samples were placed in RNA later and stored prior to extraction of RNA.

Infusion of octreotide was administered to patients with large (>2cm) gastric ECL tumours over 72h (25μg/h). Plasma samples and gastric corpus biopsies were taken before and after treatment.

The study was approved by the Ethics committees of the South Sefton and the Royal Liverpool and Broadgreen University Hospitals NHS trusts. All patients gave informed written consent. The appropriate Home Office licenses were obtained in order to carry out animal work.

Mouse and human plasma samples were assayed for total amidated gastrin concentration as described previously (McCaig *et al.* 2006).

3.2.2 Q-PCR Primer and probe optimisation

All mouse and human TIMP Q-PCR primers and probes were obtained from Eurogentec. All TIMP probes were 5' FAM labelled and 3' TAMRA labelled (for primer and probe sequences see Chapter 8.3). Mouse TIMP primers were made up to 100μM stock in TE, and working solutions of 10μM made up in 10mM Tris. Mouse TIMP probes were made up to 20μM stock solutions in TE and working solutions of 5μM were made up in 10mM Tris. Stock solutions were stored at -20°C and working solutions stored at 4°C. Human primer and probe sets were prepared to the same concentrations; however sterile distilled water rather than TE/Tris was used to make the dilutions.

Reaction concentrations of primers and probe for mouse TIMP-1 and -3 were optimised using cDNA derived from mouse kidney and TIMP-4 primers and probe were optimised using cDNA derived from mouse heart.

The human TIMP-1 and -3 primers used generate amplicons within a single exon and were therefore optimised using human genomic DNA (Promega). The human TIMP-2 and -4 primers both span and exon-exon junction were therefore optimised using cDNA derived from HeLa cells. For optimisation of primers, varying combinations of forward and reverse primer concentrations (50-900nM) were used (Table 1) and that giving the lowest Ct value adopted. Using the optimised primer concentrations the concentration of probe providing the lowest Ct value was then determined (Table 2).

No of replicates	4								
Primer combination	50F/50R	50F/300R	50F/900R	300F/50R	300F/300R	300F/900R	900F/50R	900F/300R	900F/900R
	volume (µl)								
Master mix	50	50	50	50	50	50	50	50	50
forward P	0.5	0.5	0.5	3	3	3	9	9	9
reverse P	0.5	3	9	0.5	3	9	0.5	3	9
probe	4	4	4	4	4	4	4	4	4
DNA	4	4	4	4	4	4	4	4	4
Water	41	38.5	32.5	38.5	36	30	32.5	30	24
total	100	100	100	100	100	100	100	100	100

Table 1: Primer optimisation template. The volumes shown provide master mixes to give 4 replicates. 9 different forward and reverse primer combinations are used varying between 50-900nM of each primer.

	PROBE CONCENTRATION									
	25	50	75	100	125	150	175	200	225	
Number of replicates		4								Total
	Volume (µl)									
mastr mix	50	50	50	50	50	50	50	50	50	450
primer F	3	3	3	3	3	3	3	3	3	27
primer R	3	3	3	3	3	3	3	3	3	27
probe	0.5	1	1.5	2	2.5	3	3.5	4	4.5	22.5
DNA	4	4	4	4	4	4	4	4	4	36
water	39.5	39	38.5	38	37.5	37	36.5	36	35.5	337.5
total	100	100	100	100	100	100	100	100	100	900

Table 2: Probe optimisation template, shown for 4 replicates. Concentrations of probe are increased from 25-225nM in 9 reactions, previously determined optimal primer concentrations are used.

3.2.3 Q-PCR assays

In assays performed on mouse samples, gene expression in the test samples was compared to 18S RNA abundance as an endogenous control. The 18S kit (FAM-

TAMRA labelled) was supplied by Eurogentec. Mouse samples were all diluted 1:10 for measurement of 18S. In assays performed on human samples, gene expression was compared with both 18S RNA abundance and GAPDH abundance (GAPDH primers and probe; Yakima yellow-dark quencher, Eurogentec). Samples were diluted 1:100 for 18S measurement.

A 2x master mix (Eurogentec) containing Uracil-N-glycosylase (UNG), to prevent carry over contamination was used for most Q-PCR reactions. For the assays on human samples with TIMP-2 and -4 a mastermix without UNG was used, which provides greater sensitivity.

For measuring 18S RNA abundance each reaction contained; 50µl 2x master mix (Eurogentec), 6µl primer mix (600nM), 2µl probe (125nM), 4µl DNA and 38µl water. This gave a total volume of 100µl and three 25µl replicates were used in the assay.

For each sample measured with GAPDH the reaction contained 50µl 2x master mix (Eurogentec), 2µl of each primer (200nM), 2µl of each, probe (100nM), 4µl DNA and 40µl water to make up to 100µl.

Mouse genomic DNA (Promega) was used to construct a standard curve for 18S in the reactions using mouse samples. Four serial dilutions of neat, 1:10, 1:100 and 1:1000 were used to construct this standard curve (concentration of neat genomic DNA 100ng/µl). Human genomic DNA (Promega) was used to construct 18S and GAPDH standard curves in assays using human sample. The dilutions used were neat, 1:10, 1:100, 1:1000 and 1:10000 (concentration of neat genomic DNA 100ng/µl).

For experiments using FAM-TAMRA labelled primers and probes, (mouse and human TIMP and 18S), detection was performed using the FAM dye layer with

TAMRA as the quencher. The GAPDH primers and probes were Yakima yellow-dark quencher labelled and detection was performed using the VIC dye layer, with no quencher.

In assays measuring TIMP expression in mouse samples mouse heart cDNA was used to construct the standard curves. Dilutions of 1:50, 1:100, 1:250 and 1: 1000 were used for TIMP-3 and dilutions of 1:5, 1:10, 1:25 and 1:100 for TIMP-2 and -4 (concentration of undiluted heart cDNA 1.83μg/μl). In assays measuring TIMP expression in human samples, human genomic DNA (Promega) was diluted 1:100, 1:1000, 1:10 000, 1:100 000 for TIMP-3 and 1:100, 1:400, 1:2000, 1:10 000 and 1:50 000 for TIMP-1. For assays to measure human TIMP-2 and -4, HeLa cell cDNA was used neat and diluted 1:10, 1:100 and 1:1000 (concentration of undiluted HeLa cDNA 1.9μg/μl).

3.3 Results

3.3.1 Optimisation of TIMP primers and probes for q-PCR

Mouse TIMP primer and probe optimisation determined that the quantities shown in table 3 gave the lowest Ct values. These values were used for all reactions to measure TIMP expression in mouse samples, to give optimal results.

	mTIMP1	mTIMP3	mTIMP4
	Volume (µl)		
Master Mix	50	50	50
Primer F	3	9	9
Primer R	9	3	3
Probe	4.5	3.5	4
DNA	4	4	4
SDW	29.5	30.5	30
total	100	100	100

Table 3: Volumes (µl) of reagents, determined by optimisation experiments, added per sample for Q-PCR assays on mouse cDNA samples. Working concentration of primers 10µM and probes 5µM.

Human TIMP primer and probe optimisation determined that the quantities shown in table 4 gave the lowest Ct values. These values were used for all reactions to measure human TIMP expression to give optimal results.

These values give a total of 100µl, this is used to give three 25µl replicates for the Q-PCR assay.

	hTIMP1	hTIMP2	hTIMP3	hTIMP4
	Volume (µl)			
Master Mix	50	50	50	50
Primer F	3	3	3	3
Primer R	9	3	3	3
Probe	4	4	4.5	4
DNA	4	4	4	4
SDW	30	36	35.5	36
total	100	100	100	100

Table 4: Volumes (µl) of reagents, determined by optimisation experiments, added per sample for Q-PCR assays on human cDNA samples. Working concentration of primers 10µM and probes 5µM.

The optimisation assays for each primer set and probe allowed determination of which combination of primer concentrations and which probe concentration gave the lowest Ct value. This indicated the highest assay sensitivity. These concentrations were used in all subsequent assays in order to achieve optimal results. An example of a result from a primer and probe concentration optimisation experiment is shown in Fig 2. The optimal combinations for each primer set and probe are shown in table 5.

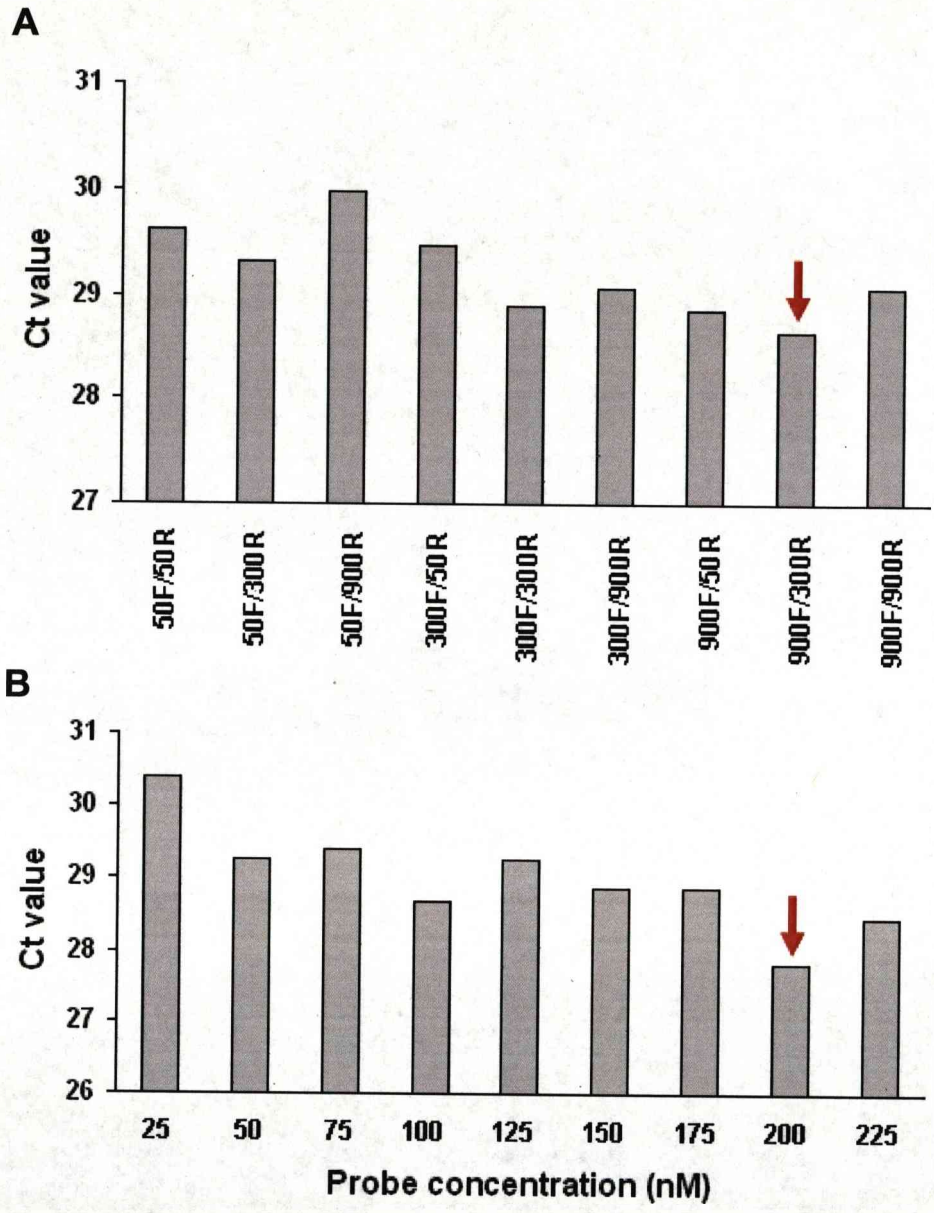


Fig 2: One example of primer and probe optimisation result (mouse TIMP-4). A. The primer combination giving the lowest Ct value is chosen, in this case 900nM forward primer and 300nM reverse primer. B. The probe concentration giving the lowest Ct value is chosen, in this case 200nM.

TIMP	Primer Concentrations (nM)	Probe Concentration (nM)
Mouse TIMP 1	300F/900R	225
Mouse TIMP 3	900F/300R	175
Mouse TIMP 4	900F/300R	200
Human TIMP 1	300F/900R	200
Human TIMP 2	300F/300R	200
Human TIMP 3	300F/900R	225
Human TIMP 4	300F/300R	200

Table 5: The concentrations of forward and reverse primers, and probes, used for TIMP Q-PCR assays.

3.3.3 TIMP expression is increased in Ins-GAS mice 6 months after *H. felis* infection

Q-PCR was carried out to investigate TIMP expression in the gastric corpus of Ins-GAS mice. There was an increase in TIMP-1 and -3 expression compared to uninfected control Ins-GAS mice after six months infection with *H. felis*. Three months after infection there was no difference in TIMP expression between the two groups (Fig 3). The expression of TIMP-4 was below the level of detection using this method.

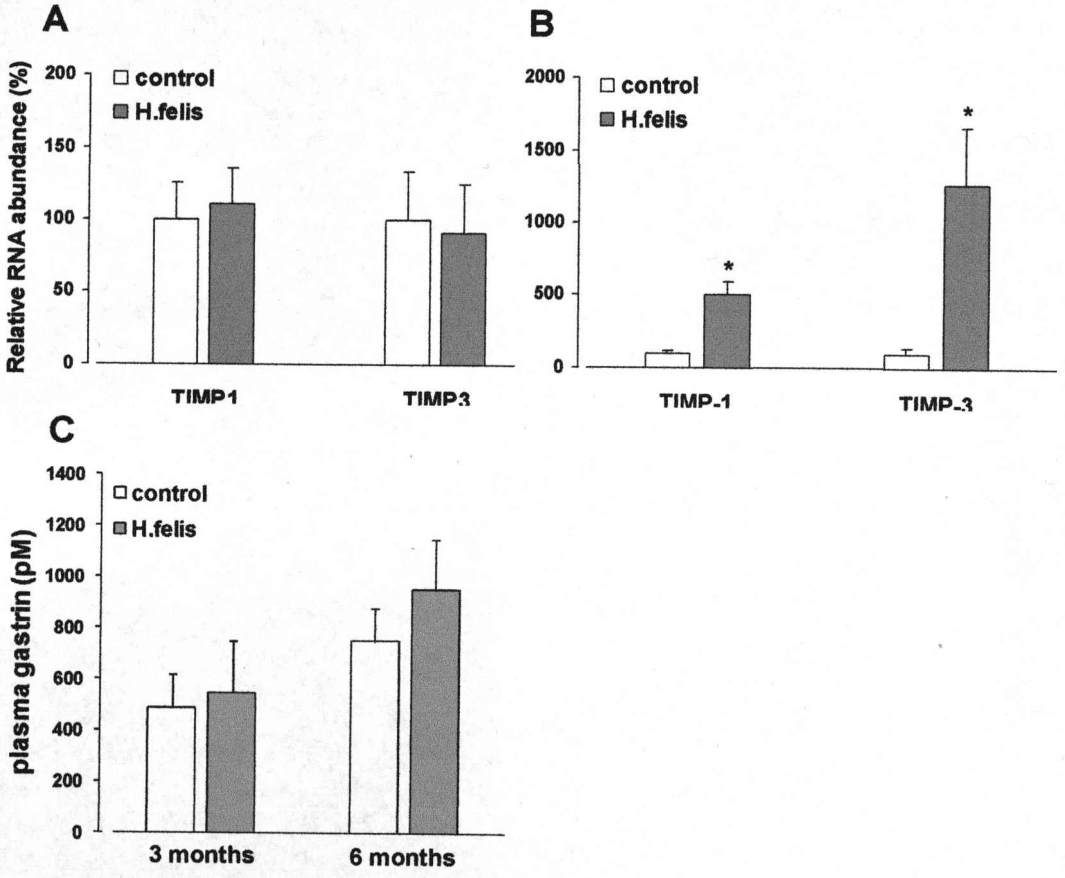


Fig 3: Q-PCR on gastric corpus samples from Ins-GAS mice infected or non-infected with *H. felis*. A. Three months after *H. felis* infection there was no change in TIMP-1 or -3 expression in Ins-GAS mice, B. 6 months after *H. felis* infection expression of TIMP-1 and -3 increased in infected mice compared to the non-infected controls. TIMP values normalised to 18S and expressed as percent of uninfected controls ± SE. C. At 3and 6 months there was no significant difference in plasma gastrin levels. Control, n = 5; *H felis*, n = 5. *$p<0.05$ Student's t-test.

3.3.4 TIMP expression is increased in patients with pathologically elevated gastrin due to PA.

Since gastrin is known to affect gastric epithelial architecture Q-PCR was carried out on human corpus biopsies from patients with pathologically high gastrin due to PA. The results showed that expression of all TIMPs increased significantly in this patient group, compared to patients with normal gastrin concentrations (<40pM) (Fig 4).

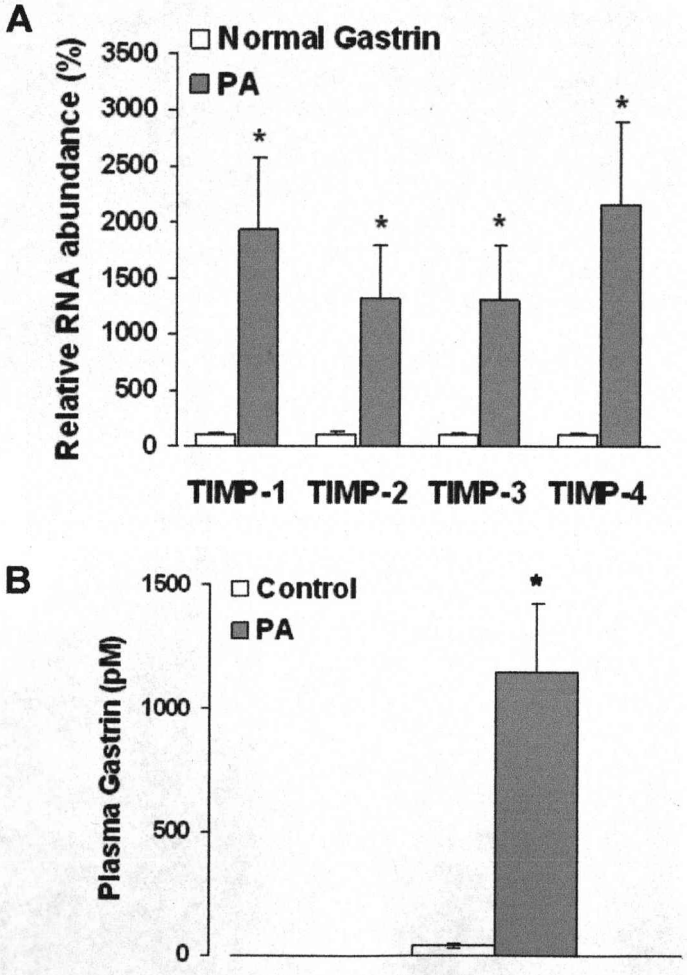


Fig 4: TIMP expression was increased in patients with elevated circulating gastrin due to PA. A. Expression of TIMPs 1-4 was increased in PA patients compared to patients with normal gastrin levels. C. Typical circulating gastrin concentration (pM) in PA patients compared to control unaffected patients. TIMP values normalised to GAPDH and expressed as percent of controls ± SE. Control, n = 8-15; PA, n = 13-16. *$p<0.05$ Student's t-test.

3.3.5 Hypergastrinaemia induced TIMP overexpression in PA is reduced following antrectomy and somatostatin analogue treatment

In patients with very high gastrin levels associated with PA, TIMP expression in gastric corpus was increased (Fig 4). When two such patients were treated with octreotide, a somatostatin analogue, for 72h both patients showed a reduction in TIMP expression. Moreover after undergoing an antrectomy to permanently reduce the gastrin drive TIMP expression was reduced by almost 50% in two further patients (Fig 5).

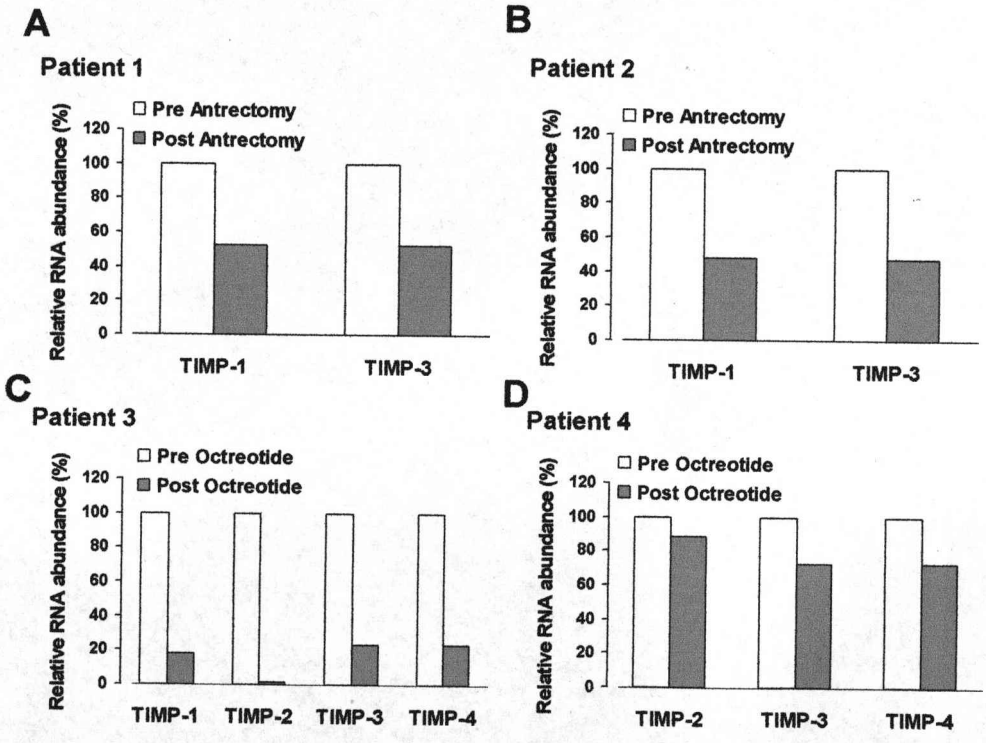


Fig 5: Reduced TIMP expression after octreotide infusion and antrectomy. (A) and (B) In 2 patients with TIMP overexpression due to PA, expression of TIMP-1 and -3 was reduced following antrectomy. (C) and (D) Following octreotide treatment TIMP expression also reduced in two other PA patients. In all cases TIMP expression was normalised to GAPDH in the same sample. Gastrin concentrations (pM): patient 1 pre 2800, post 112; patient 2 pre 1080, post 24; patient 3 pre 680, post 215; patient 4 pre 3400, post 490. (n=1).

3.4 Discussion

Before starting to perform Q-PCR reactions all the TIMP primer and probe sets intended for use required optimisation, to ensure maximum assay sensitivity was achieved. This entailed finding a suitable cDNA to use in the optimisation assays, and where necessary, for standard curves. In order to optimise the mouse TIMP primer sets mouse heart, liver and kidney cDNA were initially used. These tissues were chosen because all were easy to obtain and also expressed TIMPs to an adequate level. The best results were obtained using kidney cDNA for mouse TIMP-1 and -3 and mouse heart cDNA for TIMP-4. The appropriate tissue was chosen by running real time PCR assays with the TIMP primers and probes to discover which cDNA gave the best amplification (lowest Ct value). Mouse heart cDNA was subsequently used to generate a standard curve in assays to measure all three TIMPs, although kidney cDNA was used to optimise the TIMP-1 and 3 primers and probes. The reason for this was that there was not a great deal of difference between the results obtained with kidney or heart cDNA and by using heart the standard curves generated in all assays on the mouse samples used the same cDNA, from a single reverse transcription.

The human TIMP-1 and -3 primers and probes could be optimised using human genomic DNA because these primers did not span an exon-exon boundary. However the primers for human TIMP-2 and -4 did span an exon-exon boundary and therefore another source of DNA needed to be used for these optimisations. A suitable source of cDNA was found in a similar way as for the mouse assays. cDNA was prepared from human cell lines, HeLa, CaCo2 (a human colon cancer cell line), and AGS-G_R

cells (a human gastric cancer cell line). Q-PCR assays were carried out with the TIMP primers and probes to discover which cDNA gave the best amplification (lowest Ct value). The best results were seen with HeLa cell cDNA and this was subsequently used for optimisation and also for standard curves in assays measuring human TIMP-2 and -4 expression.

Expression of all mouse TIMPs was compared to expression of 18S RNA in the same sample, to give a ratio for TIMP expression. Initially 18S expression was also determined for each human sample but it was found, however that for the human samples, standardization against GAPDH provided more consistent data.

Expression of TIMP-1 and -3 was increased in Ins-GAS mice, six months after infection with *H. felis*, compared to the non-infected control mice. There was no difference in TIMP expression three months after infection; in fact TIMP expression was very low in all samples prior to 6 months. TIMP-4 expression was investigated in the same samples, however the expression was too low to detect by q-PCR. A reason for this may be a variation in the sensitivity of the primers and probe. TIMP-2 expression was not investigated in the Ins-GAS mice because the preliminary data using human samples had shown no difference in TIMP-2 expression with *H. pylori* infection.

These results from the mouse samples are interesting as they are similar to those seen when investigating TIMP expression with *H. pylori* infection in humans. In both humans and mice *Helicobacter* infection induces overexpression of TIMP-1 and -3. It is useful to have an animal model for investigation of TIMP expression in response to *Helicobacter* infection, using an animal model will enable more control

over experimental factors such as age, weight, environment and time of infection and thus reduce variability.

Ins-GAS mice have raised plasma gastrin concentrations. They show increased proliferation in the gastric mucosa and, after 4 months of age, loss of parietal cells with subsequent decrease in acid secretion. The mice develop hyperplasia, similar to that seen in humans with CAG. Older mice have an increased tendency to develop gastric cancer; this is accelerated with *Helicobacter* infection (Wang *et al.* 1996, Wang *et al.* 2000). Gastrin levels in the Ins-GAS mouse samples used were similar in the control and infected mice. This suggests that in these experiments inflammation due to *H. felis* infection rather than hypergastrinaemia was responsible for the increase in TIMP expression.

TIMP expression may increase during *Helicobacter* infection, to compensate for the increase in the expression of MMPs, for example, MMP-7 expression was increased in gastric epithelial cells following *H. pylori* infection (Wroblewski *et al.* 2003). Presumably, MMP expression increases with infection due to host response to inflammation and remodelling of the ECM. Alternatively TIMP expression could increase for reasons independent of MMP inhibition.

The results show that expression of all four TIMPs increased significantly in gastric biopsy samples from patients with PA, compared to samples from patients with normal gastrin levels (<30pM). In PA, circulating gastrin levels become extremely high, and the condition develops over many years, these patients could have circulating gastrin concentrations of 2000pM, over 10 times higher than normal, persisting over a period of several years. There was no significant increase in TIMP expression between patients with normal gastrin levels and those with elevated

gastrin due to conditions other than PA, such as gastrinoma or treatment with proton pump inhibitors (PPIs) (data not shown). PPIs are not likely to result in such high gastrin levels as those seen in PA, and this would occur over a shorter period of time. Gastrinoma may result in very high gastrin levels, although as with PPIs this would be present over a shorter time period. This suggests that very high gastrin levels, or that elevated gastrin over a very long period of time is required to increase TIMP expression.

Another interesting point arising from the data is that although *Helicobacter* infection did not increase TIMP-2 expression in humans (Fig. 2 and Bodger *et al.* 2008), elevated gastrin in PA did increase its expression. *H. pylori* infection is associated with gastric cancer (Uemura *et al.* 2001), and it is possible that hypergastrinaemia could exacerbate the effects of inflammation due to this infection, which is known to cause an increase in gastrin concentration, although not to such high levels as seen in PA (Calam *et al.* 1997). All the patient samples used in this work were *H. pylori* negative.

In the normal condition, acid from the parietal cells stimulates release of somatostatin from D-cells in the antrum, which inhibits gastrin release from G-cells. In PA there is loss of parietal cells therefore no somatostatin is released, this leads to hypergastrinaemia and commonly ECL cell hyperplasia (Jensen 2002) (fig 6). Also elevated gastrin leads to the induction of ECL cell gene expression, such as histidine decarboxylase (HDC), chromogranin A (CGA), vesicular monoamine transporter type 2 (VMAT2) (Dimaline *et al.* 1993a , Dimaline *et al.* 1993b, Dimaline and Struthers 1996), and also Reg 1α, which may function as a tumour suppressor (Higham *et al.* 1999).

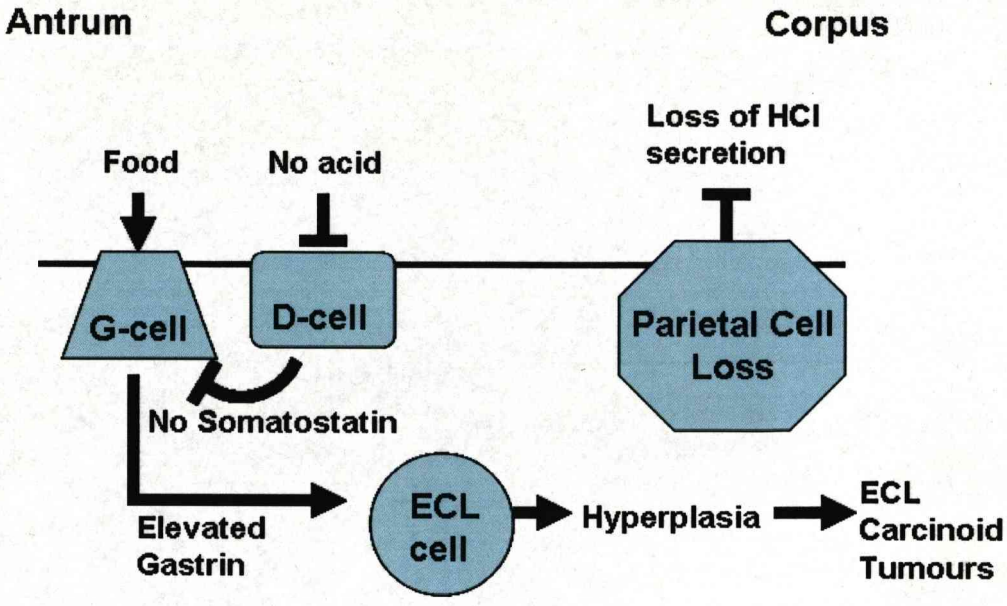


Fig 6: A schematic representation of the development of hypergastrinaemia in PA. In the normal stomach G-cells release gastrin in response to food intake. Gastrin causes ECL cells to release histamine which stimulates acid secretion from parietal cells, this feeds back on D-cells which release somatostatin to inhibit further gastrin release (see Ch1 Fig 2). In PA there is loss of parietal cells, and therefore acid release. There is no somatostatin feedback and therefore gastrin production becomes elevated leading to ECL cell hyperplasia and in some cases the development of ECL carcinoid tumours.

In 5-10% of patients with PA, ECL hyperplasia progresses to the development of gastric carcinoid tumours (Bordi *et al.* 1991, Bordi *et al.* 1995), the majority of these tumours are small (<1cm) and are rarely metastatic (Solcia *et al.* 1991, Modlin *et al.* 1995), however larger carcinoid tumours (>2cm) carry a significant risk of leading to metastasis (Thomas *et al.* 1995). Antrectomy has been performed to treat small ECL tumours, by removing the gastrin secreting G-cells located in the antrum. However when larger tumours are present total gastrectomy has been advised due to the risk of metastasis (Hirschowitz *et al.* 1992, Thomas *et al.* 1994).

Octreotide is a somatostatin analogue and is known to decrease plasma gastrin levels and reduce ECL cell number in patients with chronic atrophic gastritis (Bordi *et al.* 1993, Ferraro *et al.* 1996). An octreotide suppression test, as described by Higham *et al.* (1998), was used to determine whether large carcinoid tumours were responsive to gastrin, and thus whether antrectomy would be an appropriate treatment. Infusion of octreotide over 72h resulted in the temporary reduction of plasma gastrin levels, and depression in ECL cell function (assessed by gene expression), indicating that these cells were responsive to gastrin and that antrectomy would be therapeutically beneficial. Indeed antrectomy, performed 3 months following the suppression test, resulted in the regression of the ECL carcinoid tumours. The octreotide suppression test is therefore a useful tool to determine whether antrectomy, rather than total gastrectomy, is an appropriate treatment for large ECL carcinoid tumours.

Samples were obtained from patients with hypergastrinaemia due to PA after 72h octreotide infusion, and also pre and 1-3 years post antrectomy. These data showed that antrectomy caused a reduction in TIMP expression in both patients tested. In two other PA patients TIMP overexpression was reduced after octreotide supression tests. The results suggest that upregulation of TIMP expression due to PA is reversed after long term reduction of gastrin secretion by antrectomy.

In conclusion the results shown in this chapter indicate that TIMP expression in the gastric epithelium increases with *Helicobacter* infection in Ins-GAS mice and humans. Also TIMP expression increases in the corpus of the stomach in patients with elevated gastrin due to PA. This upregulation is reduced by performing an antrectomy which lowers circulating gastrin levels.

Chapter 4

Expression, regulation and function of TIMPs in response to growth factors and gastrin

4.1 Introduction

Interactions between epithelial cells and mesenchymal cells are very important in maintaining normal mucosal organisation. This occurs during normal physiological processes such as development and wound healing, but also in pathology such as progression to cancer.

Epithelial and sub-epithelial cells may release growth factors, cytokines and extracellular proteases, such as MMPs, and protease inhibitors, as a way to communicate with each other, and alter the microenvironment.

Examples of dysfunction in this system are *H. pylori* infection which causes inflammation of the gastric mucosa, and in some patients leads to gastric cancer (Correa & Chen 1994), and also prolonged hypergastrinaemia in patients with pernicious anaemia (PA) leading to ECL cell carcinoid tumours (Bordi *et al.* 1995).

Myofibroblasts are an important sub-epithelial cell type due to their ability to produce epithelial growth factors and their close proximity to the epithelial cells.

Previous work carried out in this laboratory has shown that *H. pylori* infection increases the production of MMP-7 by gastric epithelial cells (Wroblewski *et al.* 2003). MMP-7 acts as a myofibroblast growth factor by promoting the cleavage of IGFBP-5, secreted by myofibroblasts, to liberate IGF-II, which stimulates gastric epithelial cell proliferation and myofibroblast proliferation and migration. Over time increased numbers of myofibroblasts can lead to hyperproliferation and increase the

chance of developing malignant transformations (Hemers *et al.* 2005, McCaig *et al.* 2006).

Immunohistochemistry (IHC) performed on endoscopic biopsy samples from *H. pylori* positive gastric corpus mucosa to determine cellular localisation of TIMPs in the gastric mucosa showed differences in the localisation of TIMP-1, -3 and -4. TIMP-1 was localised in epithelial and inflammatory cells, and also in myofibroblasts. TIMP-3 showed strong expression in myofibroblasts, and was also present in epithelial and inflammatory cells. TIMP-4 was localised mostly to epithelial and inflammatory cells, and was not present in myofibroblasts (Fig 1 A-F). TIMPs were very weakly stained in *H. pylori* negative samples (data not shown). (Bodger *et al.* 2008).

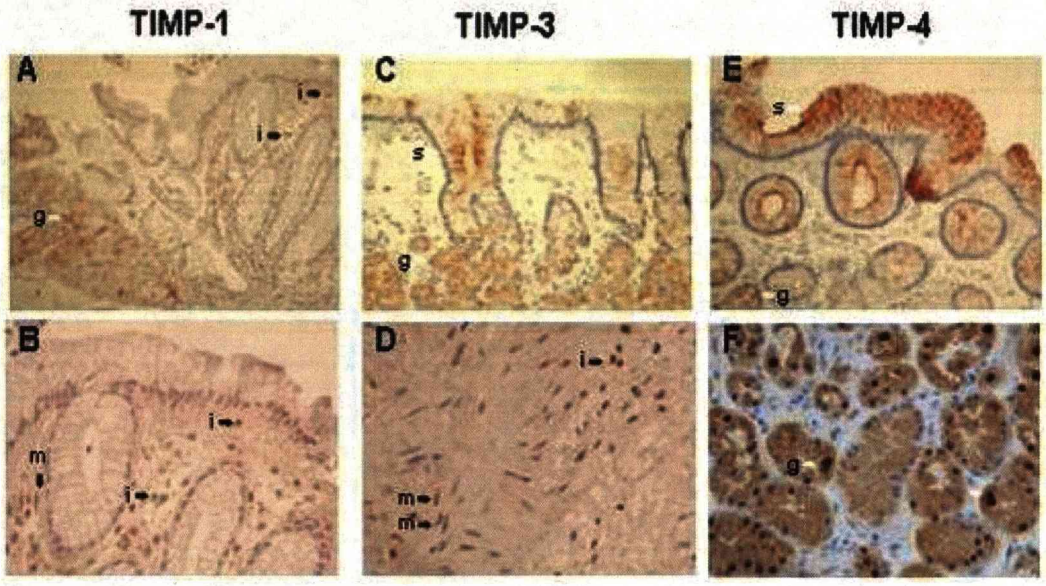


Fig 1: Immunohistochemical staining showing localisation of TIMP proteins in formalin fixed sections from *H. pylori* positive gastric corpus mucosa. A. x200 magnification image showing TIMP-1 staining in glandular epithelial cells (g) and inflammatory cells (i). B. x400 magnification image showing weak positivity for TIMP-1 in myofibroblasts (m). C. x200 magnification showing TIMP-3 positivity in surface (s) and glandular epithelial cells. D. x200 magnification showing TIMP-3 positivity in myofibroblasts and inflammatory cells. E.x200 magnification image showing TIMP-4 staining in surface and glandular epithelial cells. F. x200 magnification image showing TIMP-4 positivity in glandular epithelial cells. IHC from Bodger *et al.* 2008.

The role of MMPs in epithelial-mesenchymal signalling, and in the development of gastric cancer, makes investigating the role of their natural inhibitors, the TIMPs, in these processes relevant and important.

This chapter focuses on the expression and biological relevance of TIMPs both in gastric epithelial and sub-epithelial cells, in particular in gastric myofibroblasts and in a gastric cancer cell line, AGS-G_R.

4.2 Materials and Methods

4.2.1 Stimulation of cultured cells for protein extraction

4.2.1.1 Myofibroblasts

Cells were grown until 80-90% confluent in full serum medium (FSM) in T75 culture flasks, and then serum starved for 20-24h prior to stimulation. Cells were stimulated for 24h by adding HGF (hepatocyte growth factor) (40ng/ml), TGF-β (transforming growth factor-beta) (2.5ng/ml) or PDGF (platelet-derived growth factor) AA and BB isoforms (10ng/ml each) to the cells in 10ml serum free medium (SFM).

4.2.1.2 AGS-G_R

Cells were grown in 10cm^2 culture dishes for 48h in 10ml FSM. Cells were stimulated with 5nM G17 (Bachem) in 10ml SFM for 24h. Control (unstimulated)

cells were incubated in 10ml SFM for 24h prior to protein extraction (see chapter 2.2.2).

4.2.2 Western Blotting

80μg myofibroblast or 60μg AGS-G_R cell lysates, and 20μl each media sample from equal cell numbers, were loaded per well of a 12% SDS-polyacrylamide gel. See chapter 8.4 for antibody details and dilutions.

4.2.3 Immunocytochemistry

Myofibroblasts were seeded into 4-well chamber slides, 20,000 cells per well, and left in FSM for 24h. Cells were then serum starved for 24h before fixing in 4% paraformaldehyde and incubating with TIMP primary antibodies.

AGS-G_R cells were seeded in 4-well chamber slides at 50,000 cells per well and left in FSM for 24h. Cells were stimulated with 5nM G17 (Bachem) in 1ml SFM per well for 24h. Control (unstimulated) cells were incubated in 1ml SFM/well for 24h prior to fixing.

AGS-G_R cells transfected with TIMP-3 siRNA (Ambion, Warrington UK, details of transfection procedure in chapter 2.2.9.1) were seeded in 4-well chamber slides, approximately 20,000 cells per well, and left in FSM for 72h following transfection. Cells were then stimulated with 5nM G17 (Bachem) in 1ml SFM/well for 16h prior to fixing. See chapter 8.5 details and dilutions.

4.2.4 Transfection of AGS-G_R cells with siRNA

AGS-G_R cells were transfected with TIMP-3 siRNA (50μM), or scrambled control RNA (62μM) by nucleofection, using amaxa cell line nucleofector solution. Full details of transfection procedure in chapter 2.2.9.1

4.2.5 Boyden Chamber migration Assays

Non-transfected AGS-G_R cells were grown until confluent in T75 tissue culture flasks. AGS-G_R cells transfected with TIMP-3 siRNA (Ambion) were grown in T25 tissue culture flasks for 72h following transfection.

Migration assays were performed in 24-well 8μm pore Boyden chambers (Becton Dickinson, Oxford, UK). The Boyden chamber approach involves two chambers (Fig 2) separated by a filter through which cells migrate. Chemotactic gradients can be set up by placing the chemo-attractant in the lower chamber and the cells in the upper chamber. The cells in question migrate through the pores in the filter to enter the lower chamber. The number of cells on the lower side of the filter are then fixed, stained and counted.

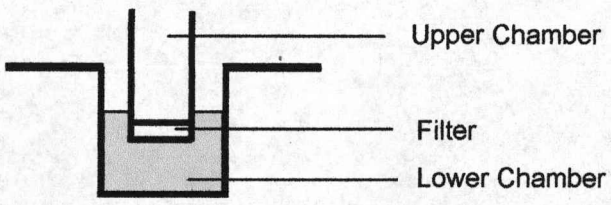


Fig 2: The Boyden Chamber. Cells are placed in the upper chamber and chemo-attractant in the lower chamber. The cells migrate through pores in the filter.

Confluent cells were detached using 0.02% EDTA and then diluted in SFM before counting.

25,000 cells were added to 0.5ml SFM/well and this was added to the wells. 0.75ml SFM was added to the lower chambers, with 5nM G17 added to the required wells. The plates were incubated at 37°C for 16h and cells migrating through the membrane were counted on the lower surface using Diff-Quick differential staining kit (Dade Behring Inc, Newark DE). Total cells in 5 fields were counted per triplicate, and the mean taken.

4.3 Results

4.3.1 TIMP-1, -2, -3 and -4 are expressed in myofibroblasts of patients with gastric adenocarcinoma

Since IHC showed expression of TIMP-1 and -3 in gastric myofibroblasts, Western blotting was performed on whole cell lysates from cultured gastric myofibroblasts. This showed that all four TIMPs were expressed in patients with gastric adenocarcinoma (Fig 3). TIMP-1, -2 and -4 expression was increased in myofibroblasts derived from the tumour, compared to adjacent normal tissue. TIMP-3 was expressed to a similar level in both sets of cells.

4.3.2 TIMP -1 and - 3 are expressed in gastric myofibroblasts

The finding that TIMP-1 and -3 were expressed in sub-epithelial cells shown by IHC, and Western blotting is consistent with the expression of TIMPs 1-4 in cultured myofibroblasts from a patient with gastric adenocarcinoma. TIMP-3 expression was also strong in myofibroblasts from adjacent normal tissue; therefore immunocytochemistry was carried out to investigate the localisation of TIMP-1 and -3 in these cultured gastric myofibroblasts (Fig 4). This shows that TIMP-1 and -3 are both expressed with a cytoplasmic localisation in myofibroblasts from the site of a gastric adenocarcinoma, and in myofibroblasts from adjacent normal tissue.

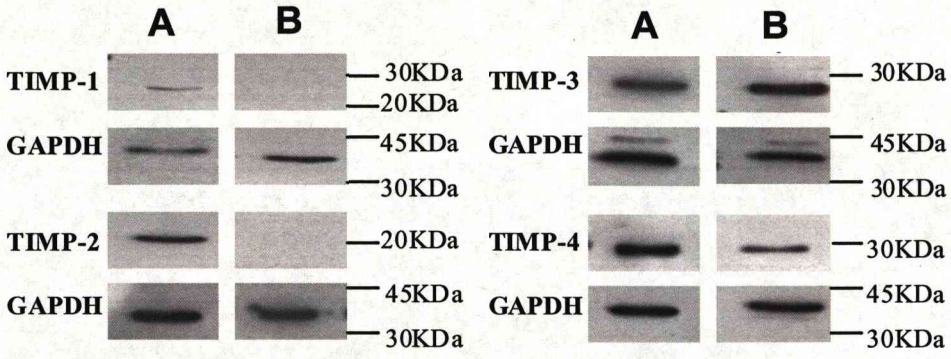


Fig 3: Western blots showing TIMP expression in myofibroblasts from a gastric adenocarcinoma. A. Lysates from cells taken from the tumour. B. Cell lysates from adjacent normal tissue. GAPDH is shown as a loading control (Representative gels from 2-3 experiments).

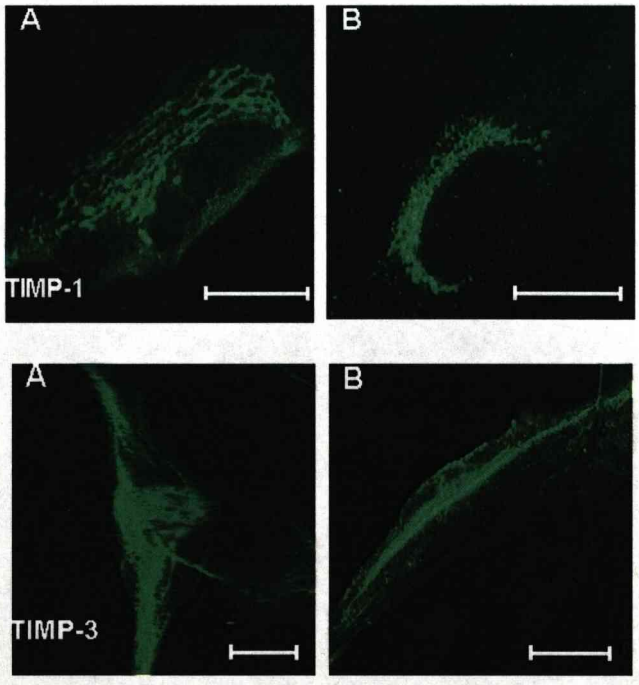


Fig 4: Immunocytochemistry showing cytoplasmic localisation of TIMP-1 and -3 in gastric myofibroblasts. A cancer derived myofibroblasts, B are myofibroblasts derived from adjacent normal tissue (Representative images from 2-3 experiments). Scale bars represent 20μm. Deconvolution was performed using AxioVision 4.6 imaging software (Zeiss, Welwyn Garden City, UK).

4.3.3 HGF and TGF-β stimulates expression of TIMP-1 in gastric myofibroblasts

To study the regulation of TIMPs in gastric myofibroblasts by growth factors Western blotting was performed. This showed that gastric myofibroblasts derived from normal gastric corpus mucosa, stimulated with HGF and TGF-β, had increased abundance of TIMP-1 in the medium compared to control cells (Fig 5B). PDGF did not alter TIMP-1 levels in the medium compared to the control. These data were normalised to the amount of total protein present in the medium (Fig 5A). TIMP-1 expression was below the limit of detection by Western Blotting in cell lysates from these myofibroblasts.

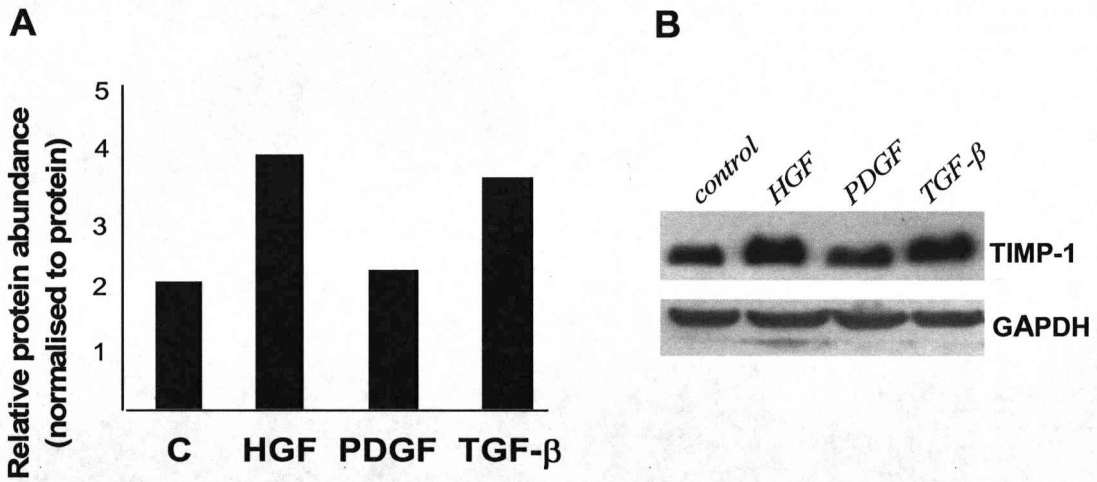


Fig 5: A. Abundance of TIMP-1 in medium from human gastric myofibroblasts from a normal patient is stimulated by HGF and TGF-β, compared to control unstimulated, cells. Relative protein abundance was measured by densitometry and normalised to the total amount of protein present in the sample (n=1). B. Representative Western Blot image showing expression of TIMP-1 in medium from gastric myofibroblasts stimulated with HGF, PDGF and TGF-β. Total amount of protein in media samples (mg/ml); control 0.78, HGF 0.74, PDGF 0.82, TGF-β 0.72.

4.3.4 HGF, PDGF and TGF-β stimulated expression of TIMP-3 in gastric myofibroblasts

Western blotting showed that stimulation of gastric myofibroblasts derived from normal human gastric corpus mucosa with HGF, PDGF and TGF-β increased expression of TIMP-3 in whole cell lysates compared to control cells (Fig 6 A and B). TIMP-3 abundance was below the limit of detection in the medium from these cells by Western Blotting.

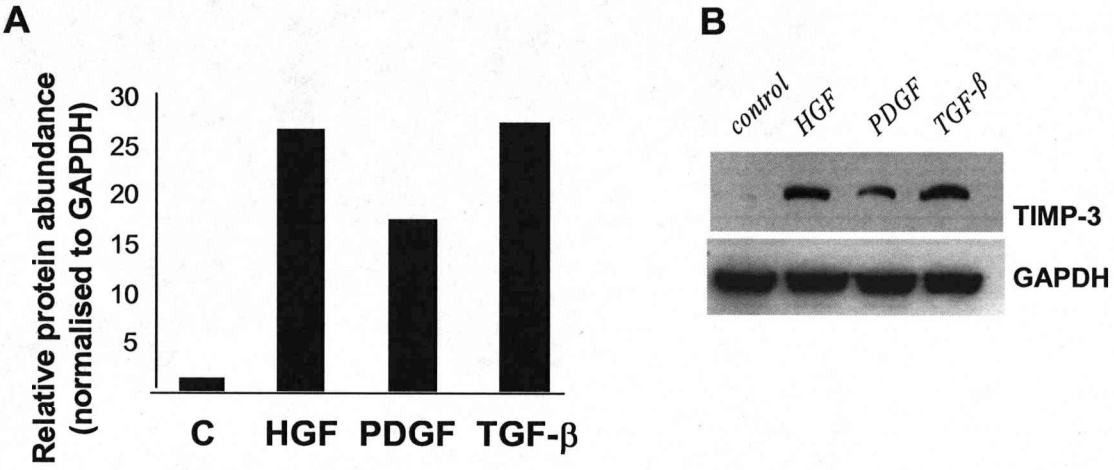
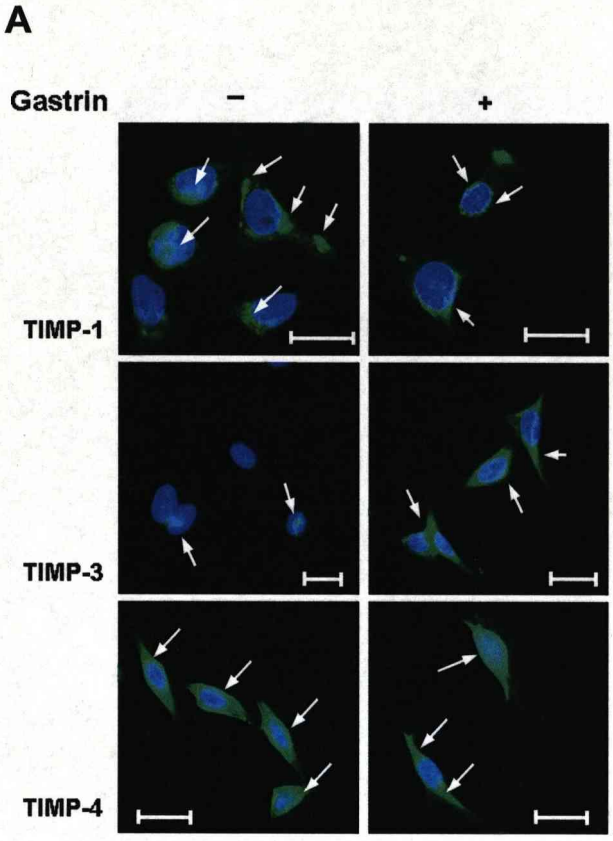


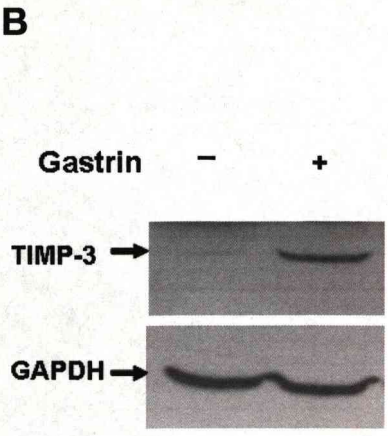
Fig 6: A. TIMP-3 expression in cell extracts from normal human gastric myofibroblasts was stimulated by HGF, PDGF and TGF-β, compared to control unstimulated cells. Data quantified by densitometry and normalised to GAPDH (n=1). B. Representative Western blot image showing expression of TIMP-3 in gastric myofibroblasts stimulated by HGF, and PDGF and TGF-β. GAPDH is shown as a loading control.

4.3.5 TIMP-1, - 3 and -4 are expressed in AGS-G_R cells but only TIMP-3 expression is induced by gastrin

TIMPs were shown by Western Blot to be expressed in myofibroblasts from the site of gastric adenocarcinoma, and Immunocytochemistry showed TIMP-1 and -3 expression in myofibroblasts from adjacent normal tissue. TIMP-1 and -3 expression was also shown to be regulated by growth factors in myofibroblasts. I then wanted to investigate the expression and regulation of TIMPs in a gastric cancer cell line, AGS-G_R.

Immunocytochemistry was performed to investigate the expression of TIMP-1, -3 and -4 in AGS-G_R cells (Fig 7A). TIMP-1, -3 and -4 were all expressed in this cell type, showing a cytoplasmic localisation. Under control, non-stimulated conditions expression of TIMP-3 was much lower than the expression of TIMP-1 and -4. Stimulation with 5nM G17 resulted in an increase in TIMP-3 expression. G17 stimulation did not appear to alter the expression of TIMP-1 or -4. A Western Blot was carried out to confirm the affect of G17 on the expression of TIMP-3 in AGS-G_R cells (Fig 7B). This showed a substantial increase in TIMP-3 expression after stimulation of the cells with 5nM G17 for 24h, compared to the non-stimulated cells.





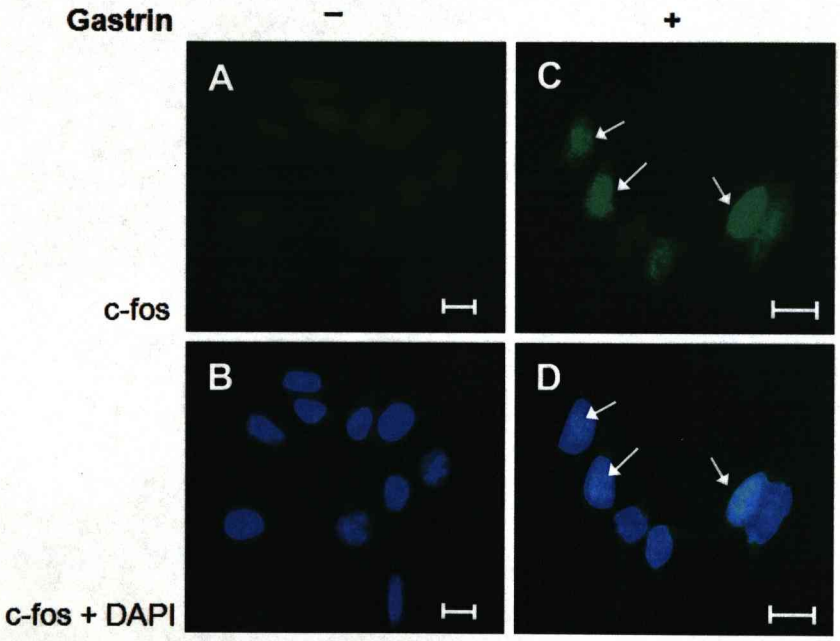
C

	Control	Gastrin
Percentage of TIMP-3 expressing cells	37.9 ± 2.9	83.3 \ 5.2

Fig 7: A. Immunocytochemistry showing staining of TIMP-1, -3 and -4 in AGS-G_R cells (indicated by arrows). The top images show TIMP-1, -3 and -4 staining in cells not stimulated with gastrin. The bottom images show cells stimulated for 8h with 1nM gastrin. The expression of TIMP-1 and -4 shows no change with gastrin stimulation but TIMP-3 expression was very low in non-stimulated cells and increased following 8h stimulation with 1nM G17 (n=3) . Scale bars represent 20μm. B. Western Blot showing upregulation of TIMP-3 expression with 24h 5nM G17 stimulation (n=2). GAPDH is shown as a loading control. C. Percentage of cells expressing TIMP-3 (measured by Immunocytochemistry). Values are percentage ± SE.

4.3.6 Gastrin induces c-fos expression in AGS-G_R cells

It is clear that TIMP-3 expression in AGS-G_R cells is stimulated by G17. Previous reports have linked gastrin stimulation to the activation of the immediate early response gene c-fos, which then induces the expression of other genes. Immunocytochemistry was performed to establish whether G17 stimulation of AGS-G_R cells induced expression of c-fos. The data show that stimulation with 5nM G17 for 2h did indeed stimulate c-fos expression in the nuclei of AGS-G_R cells. In control, unstimulated cells, no c-fos expression was seen (fig 8).



E

	Control	Gastrin
Percentage of c-fos positive cells	<1	29.8 \ 1.8

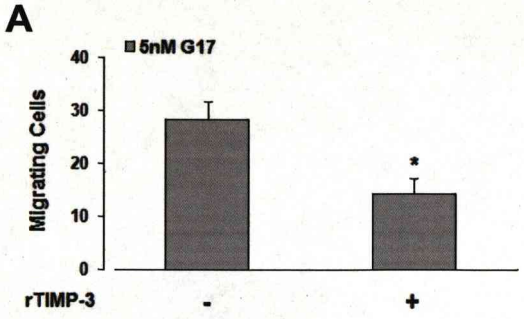
Fig 8: Immunohistochemistry showing c-fos expression in AGS-G_R cells (indicated by arrows). A and B show no c-fos staining in unstimulated cells, C and D show c-fos staining after stimulation with 5nM G17 for 2h. Scale bars represent 20μm. E. Percentage of c-fos expressing cells ± SE. n=2.

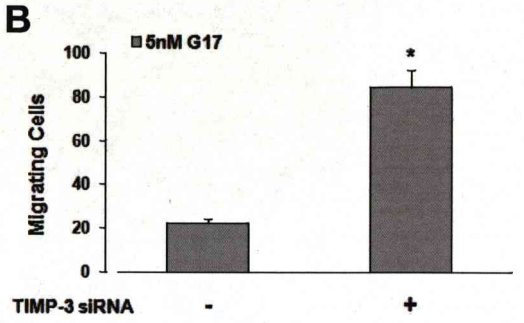
4.3.7 TIMP-3 inhibits migration of AGS-G_R cells

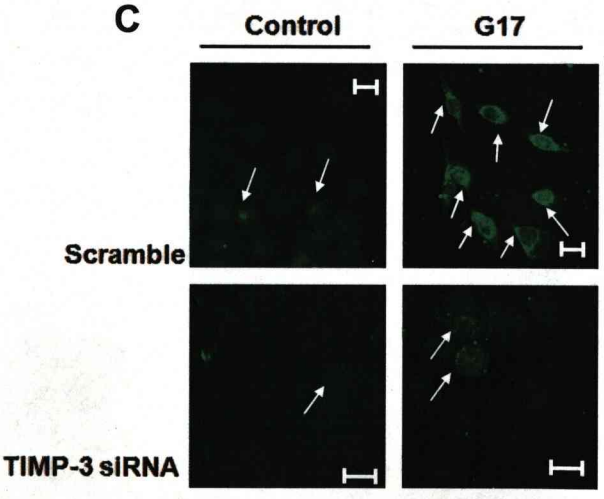
The above findings indicate that TIMP-3 is upregulated in AGS-G_R cells in response to G17 stimulation. To study the functional significance of this upregulation I went on to investigate the effect of TIMP-3 upregulation on the function of AGS-G_R cells. As I mentioned in the introduction G17 stimulates the migration of AGS-G_R cells. Incubation of AGS-G_R cells with recombinant TIMP-3 inhibited gastrin-induced migration of the cells in Boyden chamber (Fig 9A).

Knocking down TIMP-3 expression with siRNA resulted in an increase in migration of gastrin stimulated AGS-G_R cells in Boyden chamber (Fig 9B).

Immunocytochemistry verified a 54.7% knock-down of TIMP-3 expression in cells transfected with siRNA (fig 9 C & D).







D

	Scrambled		siRNA	
	Control	G17	Control	G17
Percentage of TIMP-3 positive cells	19.05 ± 2.4	78.2 ± 6.9	21.7 ± 3.7	35.4 ± 3.0

Fig 9: A. Addition of AGS-G_R cells with recombinant TIMP-3 inhibits gastrin mediated stimulation of migration through Boyden chambers. B. Knock down of TIMP-3 expression in AGS-G_R cells with siRNA stimulates migration through Boyden chambers. Cells were stimulated with 5nM G17 for 16h, results show number of migrating cells ± SE. *$p< 0.05$ Student's t-test, n=3. C. Immunocytochemistry showing knockdown of TIMP-3 in AGS-G_R after transfection with TIMP-3 siRNA. Scale bars represent 20μm. TIMP-3 indicated by arrows. n=3. D. Percentage knockdown of TIMP-3 after siRNA treatment. n=3.

4.4 Discussion

Immunohistochemistry confirmed that TIMPs are expressed in a variety of cell types in *H. pylori* positive gastric mucosa. TIMP-1, -3 and -4 showed differences in their individual cellular localisations. TIMP-1, -3 and -4 were expressed strongly in glandular epithelial cells. TIMP-3 and -4 were expressed in inflammatory cells, but TIMP-1 showed only weak positivity in these cells. TIMP-3 showed strong staining in myofibroblasts, while TIMP-1 showed weaker expression in myofibroblasts. TIMP-4 was localised exclusively in epithelial cells and not seen in myofibroblasts. This difference in cellular localisation between the individual TIMPs is interesting as it suggests varying roles for each TIMP in the gastric mucosa, and also suggests that the expression of each TIMP in the gastric mucosa is influenced in a different way by *H. pylori* infection.

Infection with *H. pylori* is known to increase the expression of MMPs, and that this is significant in the remodelling process that occurs with infection, and that may lead to gastric cancer (Wroblewski *et al.* 2003, Hemers *et al.* 2005, McCaig *et al.* 2006). Increased TIMP expression with *H. pylori* infection may be related to increased MMP expression. TIMPs also have roles that are independent of MMP inhibition (Lambert *et al.* 2004), which could also have important consequences in TIMP upregulation with *H. pylori* infection.

Myofibroblasts from a patient with gastric adenocarcinoma have been prepared and cultured in the laboratory. There are cell cultures from the site of the tumour, and from adjacent macroscopically normal tissue. Western immunoblotting on lysates

from these cells showed expression of TIMP-1, -2, -3 and -4 in cells from the tumour, and TIMP-1, -2 and -4 expression was increased in the tumour derived compared to adjacent normal tissue derived myofibroblasts.

Immunocytochemistry showed staining for TIMP-1 and -3 in the same cultured myofibroblasts, in cells from the tumour and those from adjacent normal tissue. This shows that cultured myofibroblasts do express TIMPs and therefore can be used to investigate TIMP expression and function.

Stimulation of myofibroblasts with HGF and TGF-β caused an increase in TIMP-1 abundance in the culture medium from these cells. Stimulation with HGF, PDGF and TGF-β stimulated TIMP-3 expression in whole cell lysates from myofibroblasts, showing that TIMP expression in myofibroblasts can be stimulated by growth factors.

TGF-β inhibits proliferation and therefore acts as a tumour suppressor in the early stages of cancer. However as cancer progresses cancer cells can develop resistance to TGF-β and also cancer cells can release non-physiological levels of TGF-β which may affect tumour cell differentiation thus leading to metastasis (Moustakas *et al.* 2002). Increased levels of TIMPs in myofibroblasts in response to TGF-β could be due to their roles in inhibition of ECM breakdown, or inhibition of proliferation. TIMPs have also been found to promote metastasis in some tissues (Baker *et al.* 2002).

TIMPs could also be influenced by TGF-β because of its involvement in fibrosis. An abnormally high level of TGF-β is a major factor in the development of kidney fibrosis (Moustakas & Heldin 2007).

TGF-β signalling in stromal myofibroblasts induces expression of HGF, and this promotes enhanced proliferation and invasion (Moustakas & Heldin 2007).

PDGF is a mitogen for many types of cell of mesenchymal origin and has roles in promoting cell migration and survival (Tallquist *et al.* 2004). TIMPs have roles in inhibiting cell migration (Anand-Apte *et al.* 1996, Oh *et al.* 2004) and inhibiting apoptosis (Jiang *et al.* 2002), which could explain the increase in their expression.

TIMP expression has been stimulated in other cell types by growth factors, for example TGF-β induced TIMP-1 expression in cultured intestinal myofibroblasts and myometrial smooth muscle cells, and induced TIMP-3 expression in lung fibroblasts and human chondrocytes. However TGF-β had no effect on TIMP-2 expression in myometrial smooth muscle cells (Ma *et al.* 1999, McKaig *et al.* 2003, Qureshi *et al.* 2005, Garcia-Alvarez *et al.* 2006). PDGF induced TIMP-1 expression in human dermal fibroblasts, but had no effect on TIMP-2 expression in the same cells (Jinnin *et al.* 2005).

It is interesting that the expression of the different TIMPs varies in the gastric myofibroblasts. This suggests the different TIMPs have different roles and are regulated differently in this cell type. TIMP-1 was upregulated by growth factors in media from stimulated myofibroblasts while TIMP-3 was upregulated by growth factors in the cell lysates and not in medium. This could be due to TIMP-1 and -3 having different roles in the gastric myofibroblasts. TIMP-3, unlike the other TIMPs, is tightly bound to the extracellular matrix and thus is not secreted like the other TIMPs, and would not be seen in culture medium (Woessner 2001).

From this part of the work it is clear that TIMP-1 and -3 are expressed in gastric myofibroblasts, and are regulated in these cells by growth factors. Epithelial-mesenchymal interactions are an important factor in the progression to cancer, and I went on to investigate TIMPs in a gastric cancer cell line, AGS-G_R.

AGS-G_R cells are stably transfected with the CCK2R, and respond to gastrin. Immunocytochemistry showed that TIMP-1, -3 and -4 were expressed in this cell type. TIMP-1 and -4 were expressed in unstimulated cells and in cells stimulated with gastrin; however TIMP-3 expression was very low in unstimulated cells, but was induced by gastrin stimulation. This was confirmed by Western blotting, which showed that TIMP-3 expression was increased in cells stimulated by gastrin. It has previously been shown by gene array in our laboratory that gastrin stimulates TIMP-3 expression in these cells (Varro *et al.* 2002b) and these results support this finding. These results also confirm that AGS-G_R cells can be used to investigate TIMP-1, -3 and -4 expression, and that TIMP-3 expression in these cells is particularly interesting as it is the only TIMP influenced by gastrin. In view of this, and also from previous results (chapter 1), where TIMP-3 mRNA expression in the gastric corpus was increased in *H. felis* infected Ins-GAS mice, and in patients with PA, I decided to focus future experiments on TIMP-3 regulation and function in AGS-G_R cells.

I have shown that gastrin induces c-fos expression in AGS-G_R cells. *c-fos* is part of the *Fos* (c-Fos, Fra-1, Fra-2 and FosB) family of genes, which in combination with the Jun (c-*Jun, JunB and JunD*) families, forms the AP-1 immediate early response transcription factor complex (Karamouzis *et al.* 2007). The AP-1 proteins are usually induced in response to a broad spectrum of stimuli including cytokines, growth

factors, oncogenes and stress signals. This results in the activation of various signal transduction pathways to transmit the signal to the nucleus (Young *et al.* 2006). Regulation of AP-1 is usually via changes in transcription of genes encoding AP-1 subunits, specific interactions of AP-1 proteins with other transcription factors and post-translational modification such as phosphorylation by different kinases, such as Mitogen-activated protein (MAP) Kinases (Hurd *et al.* 2002). AP-1 activity plays a central role in the control of complex biological processes like cell proliferation and differentiation (Angel *et al.* 1991).

Previous studies in our laboratory have shown that gastrin induces the expression of MMP-9 (Wroblewski *et al.* 2002) and PAI-2 (Varro *et al.* 2002b) via AP-1 activation. My results suggest TIMP-3 may be induced by gastrin in a similar manner. AP-1 has been shown to be essential for the induction of TIMP-1 gene expression by TGF-β1 (Hall *et al.* 2003), to be involved in the induction of TIMP-1 and -3 in human fibroblasts (Edwards *et al.* 1996), and TIMP-1 and -2 expression is regulated by AP-1 in breast cancer cell lines (Bachmeier *et al.* 2005).

It has been documented that gastrin stimulates the migration of AGS-G_R cells (Varro *et al.* 2002b, Wroblewski *et al.* 2002). This suggests that elevated gastrin concentration plays an important role in the disruption of normal epithelial architecture. TIMP-3 seems to inhibit the gastrin induced migration of AGS-G_R cells. This concurs with the MMP inhibitory function of TIMP-3. MMPs, for example MMP-7 and -9 are upregulated in hypergastrinaemic conditions (Varro *et al.* 2007, Wroblewski *et al.* 2002), and the increased expression of TIMP-3 seen could be a result of this increased MMP expression. Elevated TIMP-3 expression

could then act by inhibiting breakdown of the ECM by the MMPs, thus inhibiting migration.

In conclusion, this chapter shows that TIMP-1, -3 and -4 are expressed in epithelial cells in *H. pylori* infected gastric mucosa, and TIMP-1 and -3 are expressed in gastric myofibroblasts. TIMPs are also expressed in cultured gastric myofibroblasts, and their release and/or expression can be upregulated in this cell type by specific growth factors.

TIMP-1, -3 and -4 are expressed in gastric cancer cell line AGS-G_R, and TIMP-3 expression is induced by gastrin in this cell type. Gastrin also stimulates the expression of c-fos in this cell line. Investigation into the function of TIMP-3 shows that it inhibits gastrin induced migration of AGS-G_R cells.

AGS-G_R cells are a useful cell type to investigate the regulation of TIMPs, in particular TIMP-3, by gastrin.

Chapter 5

Cellular mechanisms of TIMP-3 stimulation by gastrin in AGS-G_R cells

5.1 Introduction

In the previous chapter I showed that TIMP-1, -3 and -4 are expressed in the gastric cancer cell line AGS-G_R, and TIMP-3 expression is induced by gastrin in this cell type. Also gastrin stimulated the expression of c-fos in AGS-G_R cells.

This chapter moves on to investigate the cellular mechanism by which gastrin stimulation of TIMP-3 expression occurs in AGS-G_R cells.

In addition to regulating acid secretion, gastrin has other physiological functions including an important role in the organisation and maintenance of the gastric epithelium. Gastrin upregulates the expression of several proteins important in this process, such as Reg1α (Higham *et al.* 1999), PAI-2 (Varro *et al.* 2002b), MMP-9 (Wroblewski *et al.* 2002) and MMP-7 (Varro *et al.* 2007). Also gene array data have shown that gastrin stimulates TIMP-3 expression in AGS-G_R cells (Varro *et al.* 2002b).

The mechanism by which gastrin stimulates TIMP-3 expression is relevant in order to give greater understanding into the role of TIMP-3 in gastrin mediated organisation of the gastric epithelium, in normal and pathological conditions.

The aim of this chapter is to investigate the cellular mechanism of gastrin stimulated TIMP-3 expression in AGS-G_R cells, to determine if transcriptional activation occurs, and to define regions of the TIMP-3 promoter required for responsiveness to G17 stimulation.

5.2 Materials and Methods

5.2.1 Reporter Constructs

All constructs were created using restriction enzyme digests, the DNA fragments of interest were cut from 0.8% agarose gels and cleaned using a MinElute Gel Extraction Kit (Qiagen). The promoter insert was inserted at appropriate restriction enzyme sites (Fig 2 B-E) in pGL4, transformed into competent bacterial cells, and purified. The constructs were sequenced before use.

See chapters 2 and 8.1 for detailed conditions for restriction enzyme digests, DNA ligation and elution of DNA from agarose gels.

5.2.2 Blunt ending DNA fragments

When truncating the TIMP-3-pGL4 construct to create 1.35Kb, 0.4Kb and 0.25Kb constructs, the larger construct was cut using restriction enzymes, and blunt-ended before re-ligation.

After carrying out appropriate restriction enzyme digests, the sample was cooled to 25°C and to this 1µl 2.5nM dNTP mix and 1µl klenow enzyme (Bioline) were added. This reaction was left to proceed for 20 minutes at 25°C and then heated to 75°C for 20 minutes to inactivate the enzymes.

5.2.3 Luciferase Reporter Assays

Transfected cells were stimulated in 2ml SFM after washing each well twice with SFM. Stimulation with G17 (stock 1μM), 50ng/ml EGF (stock 50μg/ml), or 100nM PMA (stock 100μM) was at 37°C for 8h in all cases apart from time course experiment. Here stimulation for 4, 8 and 24h time points were started at the same time and cells harvested after the appropriate time. For convenience the 16h stimulation was started later and carried out overnight, and cells harvested after 16h. In experiments prior to investigation into the effect of G17 concentration on TIMP-3-luc activity cells were stimulated with 1nM G17, following this experiment G17 stimulation was increased to 5nM. See chapter 2.10 for full details of assays.

5.3 Results

5.3.1 Creation of TIMP luciferase reporter constructs

Initial luciferase assays were carried out with pXP2 luciferase constructs driven by TIMP-1, -3 or -4 promoters. This gave very low luciferase readings and I decided to insert the TIMP-1 and -3 promoter inserts into pGL4 luciferase vectors. The pXP2 vector is 6.2Kb in size, compared to 4.2Kb for pGL4, and the smaller construct size can improve transfection efficiency. Moreover pGL4 demonstrates higher expression of luciferase than pXP2 since the *luc* gene is optimized for mammalian expression.

This was achieved by carrying out restriction enzyme digests to remove the TIMP promoter inserts from pXP2, and re-ligate the inserts into pGL4.

TIMP-1-pXP2 (obtained from Rod Dimaline) contained 2.6Kb of the TIMP-1 promoter. This was removed from the plasmid by cutting at Hind III and Bgl II restriction enzyme sites. The pGL4 vector was cut sequentially at Bgl II and Hind III sites, due to buffer incompatibility. The vector was cut first with Bgl II, the linearised plasmid gel-eluted and cut with Hind III. The TIMP-1 promoter fragment was then inserted at these sites (Fig 1A).

TIMP-3-pXP2 (obtained from Rod Dimaline) contained 1.6Kb of the TIMP-3 promoter. This was removed from the plasmid by cutting at SalI and BglII restriction sites. The pGL4 vector was cut at Xho I and Bgl II restriction sites. Sal I and Xho I have compatible ends and the promoter fragment was inserted at the Xho I and Bgl II sites in pGL4 (Fig 1B).

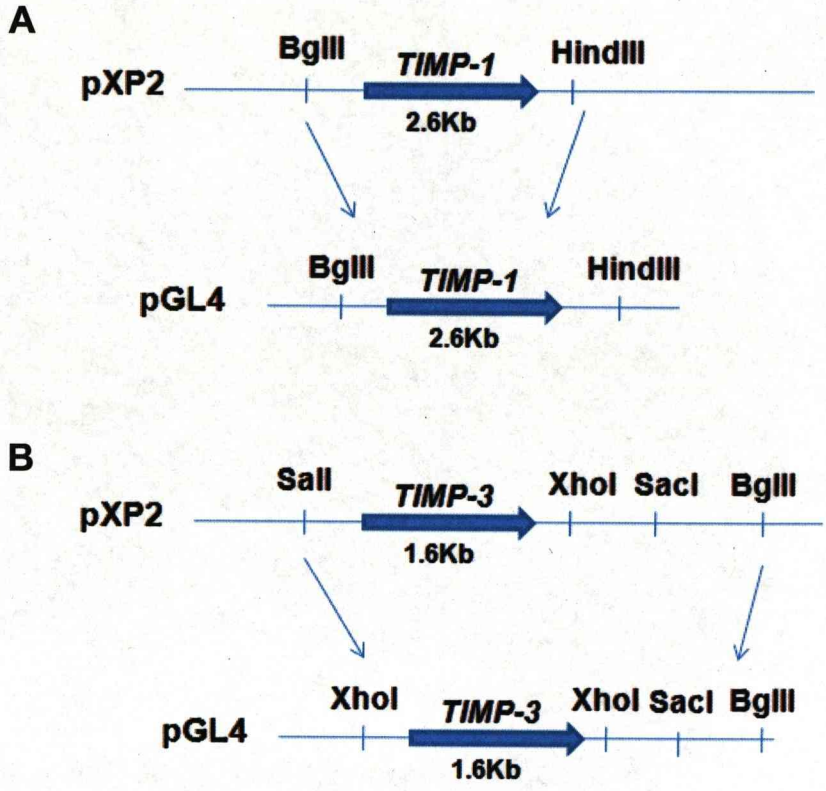


Fig 1: A. TIMP-1 promoter insert was removed from pXP2 vector at Bgl II and Hind III restriction sites. This was inserted into pGL4 at Bgl II and Hind III sites. B. TIMP-3 promoter insert was removed from pXP2 vector at Sal I and Bgl II restriction sites. This was inserted into pGL4 at Xho I and Bgl II sites.

Fig 2A shows gel purified 1.6Kb TIMP-3 promoter insert removed from pXP2 using restriction enzyme digestion with Sal I and Bgl II, and gel purified empty pGL4 vector (4.2Kb) digested with Xho I and Bgl II. The fragments were ligated, transformed into competent cells and purified using a miniprep kit (Sigma). The resulting plasmids were cut using restriction enzymes to see whether they contained

the expected TIMP promoter inserts. Fig 2B shows TIMP-3-pGL4 constructs, with the correct size insert removed by restriction enzyme digest.

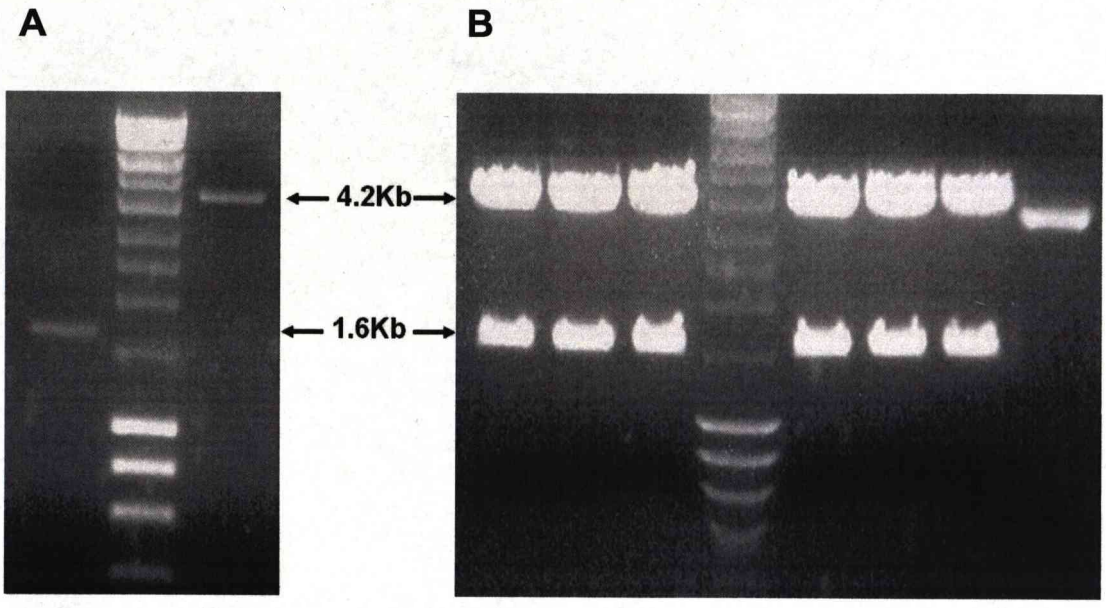


Fig 2: A. Gel purified TIMP-3 promoter insert removed from pXP2 (lane 1) and empty pGL4 vector (lane 3). B. TIMP-3-pGL4 constructs purified by miniprep and cut with Bgl I to remove the TIMP promoter insert. This gives 1.6Kb and 4.2Kb fragments. Construct number 1 (lane 1) was sequenced to ensure it contained the correct insert in pGL4 prior to large scale plasmid preparation.

In order to determine regions of the TIMP-3 promoter required for responsiveness to G17 stimulation, sequential deletions of the promoter from 1.6-0.25Kb were generated. The largest promoter construct (1.6Kb TIMP-3-pGL4) was screened for unique restricition enzyme recognition sites within the promoter fragment that could be used to generate smaller constructs containing truncated lengths of the TIMP-3 promoter. The 1.6Kb TIMP-3 promoter was inserted into pGL4 at Xho I and Bgl II restriction enzyme sites. Fig 3A shows the restriction enzyme sites available on the TIMP-3-pGL4 construct and the TIMP-3 insert. Fig 3B-E show the sites used to create the 4 further TIMP-3-pGL4 constructs. The smaller constructs were created by cutting the larger construct at the sites indicated. When creating the 0.9Kb construct, the cut construct was eluted from an agarose gel, and then re-ligated, as the construct was cut twice at Kpn I sites. When creating the other three smaller constructs (1.35Kb, 0.4Kb and 0.25Kb) the products of the restriction digest were then run on an agarose gel, the required fragment cut out and cleaned, blunt-ended and then the construct re-ligated. Fig 4 shows examples of the smaller TIMP-3-pGL4 constructs created, after being cut by restriction enzymes to remove part of the TIMP-3 promoter insert. The required fragment, containing the remaining part of the promoter in pGL4 was then cut from the agarose gel and cleaned.

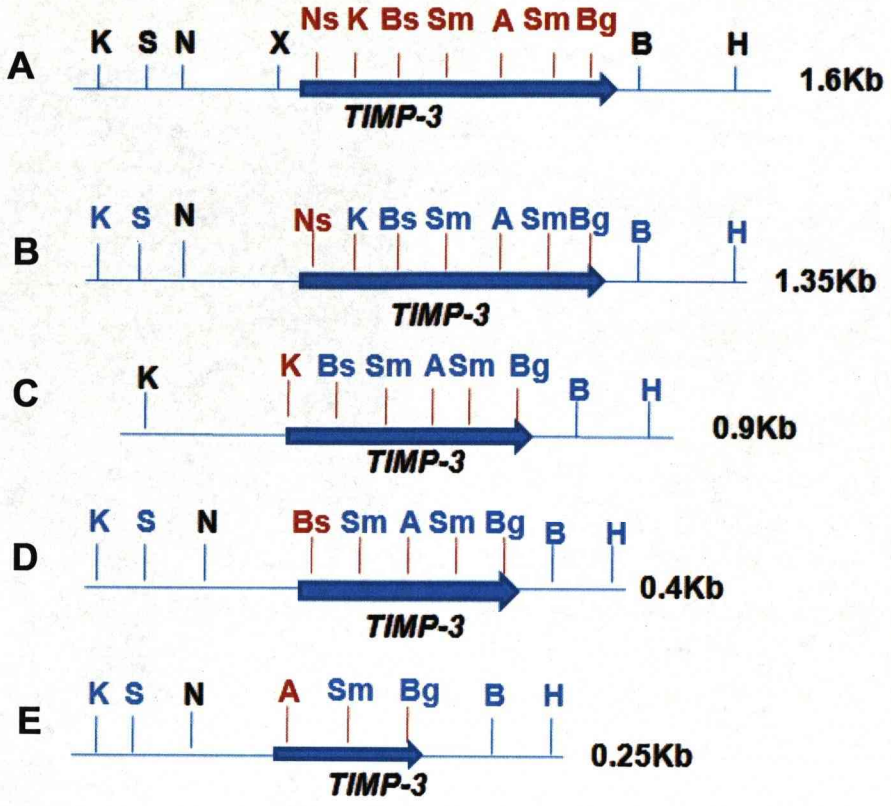


Fig 3: A. Restriction enzyme sites on pGL4: (K) Kpn I, (S) Sac I, (N) Nhe I, (X) Xho I, (B) Bgl II, (H) Hind III, and positions on TIMP-3 promoter insert: (Ns) Nsi I 234bp, (K) Kpn I 685bp, (Bs) Bsa I 1171bp, (Sm) Sma I 1244bp and 1538bp, (A) Asc I 1347bp, (Bg) Bgl I 1569bp. B-E. Sites used to create truncations of TIMP-3-pGL4 construct with sizes of insert. On B-E black represents site used to cut in pGL4, red represents site used to cut TIMP-3 promoter insert, construct was blunt-ended and re-ligated at these positions. Blue sites are those remaining on the plasmid/TIMP-3 insert.

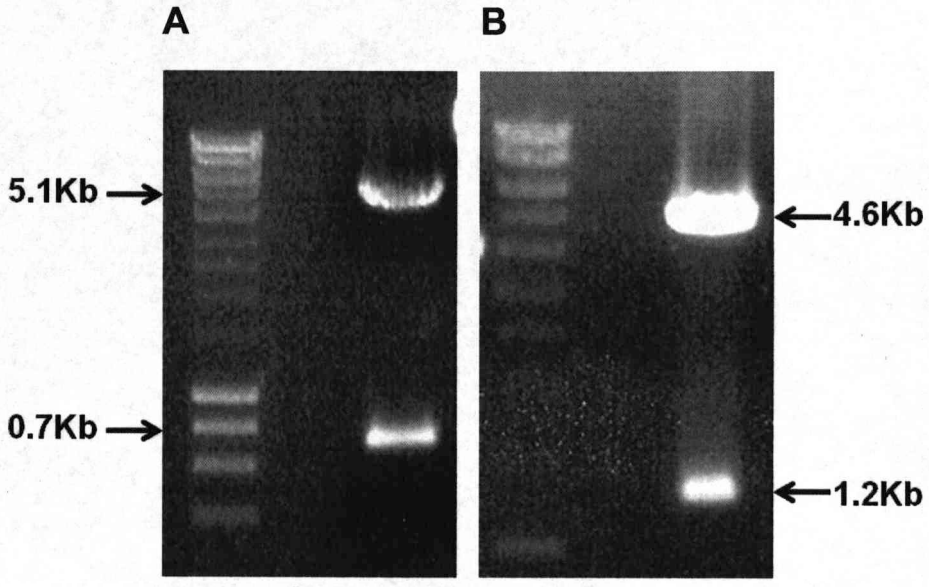


Fig 4: A. TIMP-3-pGL4 cut with Kpn I to give 0.7Kb and 5.1Kb fragments. The 5.1Kb fragment was cut from the gel and cleaned before re-ligation. This gave 0.9Kb of the TIMP-3 promoter in pGL4. B. TIMP-3-pGL4 cut with Bsa I and Nhe I to give 1.2Kb and 4.6Kb fragments. The 4.6Kb fragment was cut from the gel and cleaned before blunt ending and re-ligation. This gave 0.4Kb of the TIMP-3 promoter in pGL4.

5.3.2 Stimulation with G17 and EGF increased expression of TIMP-1, -3and -4 luc constructs in AGS-G_R cells

Previous results showed that TIMP-1, -3 and -4 are expressed in AGS-G_R cells, and that the expression of TIMP-3 is responsive to gastrin in these cells (chapter 2 Fig 6). I wanted to progress to investigate the cellular mechanism by which gastrin may stimulate TIMP expression in AGS-G_R cells. I first investigated gastrin and EGF regulation of luciferase reporter genes driven by TIMP-1, -3 or -4 promoters, in AGS-G_R cells. The results showed that the expression of each of the three luciferase-reporter constructs increased when cells were stimulated with G17 or EGF. For all three TIMPs the increase in stimulation was greater with G17 than with EGF (Fig5 A-C).

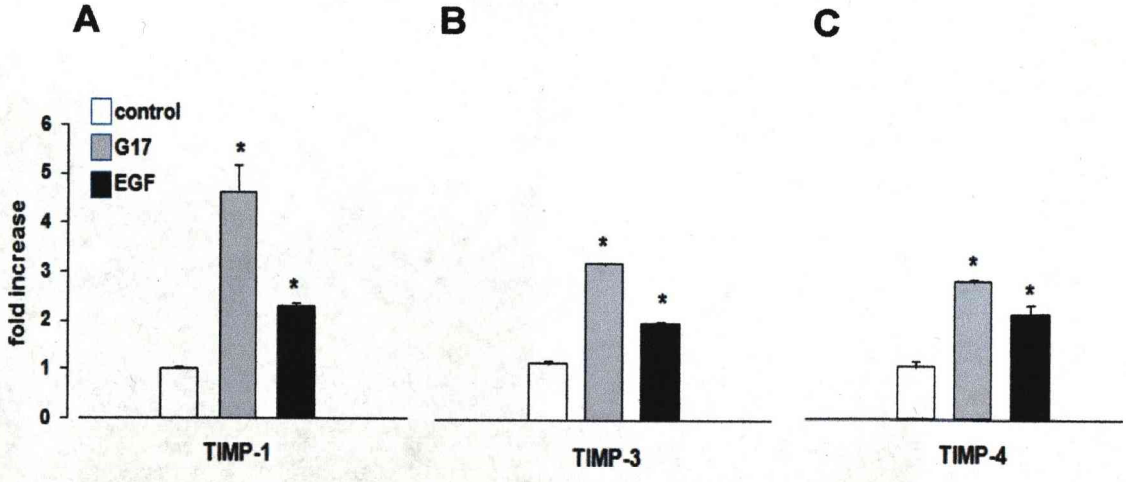


Fig 5: Expression of TIMP-1 (A), -3 (B), and -4 (C) luciferase constructs in AGS-G_R cells with G17 (1nM) and EGF (50ng/ml) stimulation for 8h. Representative results from 2-3 experiments shown. TIMP-1-pGL4, TIMP-3-pGL4 (1.6Kb), TIMP-4 pXP2 luc constructs used for these experiments. Bars show means from triplicates normalised to control ± SE. * $p < 0.05$ ANOVA, relative to control.

5.3.3 Expression of 1.6kb TIMP-3-luc in response to 1nM G17 is maximal at 16 hours in AGS-G_R cells

After carrying out initial experiments using TIMP-1, -3 and -4 luciferase constructs, which showed that G17 stimulation increased the expression of TIMP-1, -3 and -4 luciferase constructs in AGS-G_R cells, as I mentioned in the previous chapter, I decided to focus on TIMP-3 for future work. Work performed in previous chapters (Chapter 2 Figs 6&7) had shown that TIMP-3 expression was increased in AGS-G_R cells with G17 stimulation, and that TIMP-3 inhibited G17 induced migration in AGS-G_R cells. TIMP-3 had also shown interesting results in myofibroblasts where its expression was increased in cultured myofibroblasts from gastric adenocarcinoma. IHC showed strong TIMP-3 expression in epithelial and sub-epithelial cells from *H. pylori* positive gastric corpus samples from. Q-PCR showed increased expression of TIMP-3 in samples from patients with elevated circulating gastrin concentration due to PA. Considering the results from the previous work, and the fact that the results from fig 5 confirmed that G17 and EGF stimulation increased TIMP-3-luc expression in AGS-G_R cells, I decided to proceed by focusing on TIMP-3. (Chapter 2 Figs 1& 2, chapter 1 Fig 4, Bodger *et al.* 2008)

Before continuing with luciferase assays I carried out a time course experiment for G17 stimulation of AGS-G_R cells (Fig 6). This showed that the expression of TIMP-3-luc in response to G17 was maximal between 8 and 16h of stimulation. There was no difference in expression if stimulation was continued longer than 16h. However for convenience, future experiments were carried out with 8h G17 stimulation, as this also gave a significant stimulation in TIMP-3-luc activity.

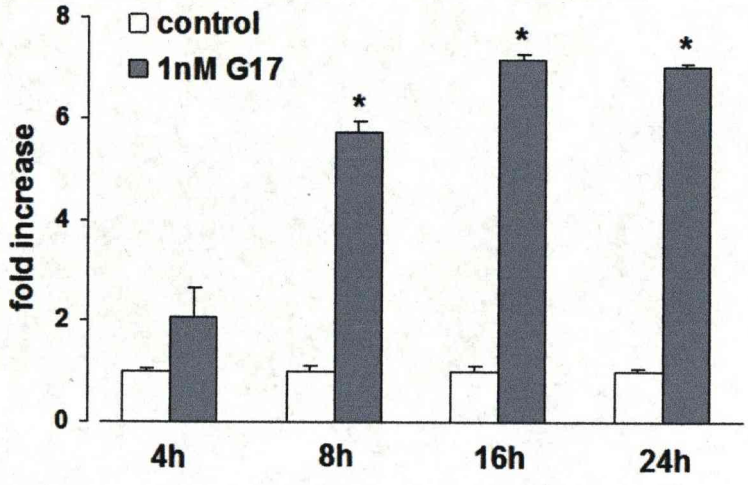


Fig 6: Time course experiment to investigate G17 stimulation of TIMP-3-luc. Expression was significantly increased after 8h stimulation, and maximal stimulation was achieved between 8-16h. There was no difference in expression after 16h stimulation. Mean of triplicates shown with values normalised to control and expressed as fold increase ± SE. *$p<0.05$ Student's t-test. (n=2).

5.3.4 Expression of 1.6kb TIMP-3-luc in AGS-G_R cells increases progressively with G17 concentration from 0.1-5nM

After carrying out a time course experiment for G17 stimulation of TIMP-3-luc expression in AGS-G_R cells, I wanted to establish the G17 concentration that gave the greatest stimulation of expression.

These experiments showed that the expression of TIMP-3-luc increased progressively with increasing concentrations of G17 over the range 0.1-5nM. There was no change in expression between 5 and 10nM G17, and 0.1nM G17 did not result in a significant increase in expression (Fig 7).

Future experiments using this construct were carried out with 5nM G17 stimulation.

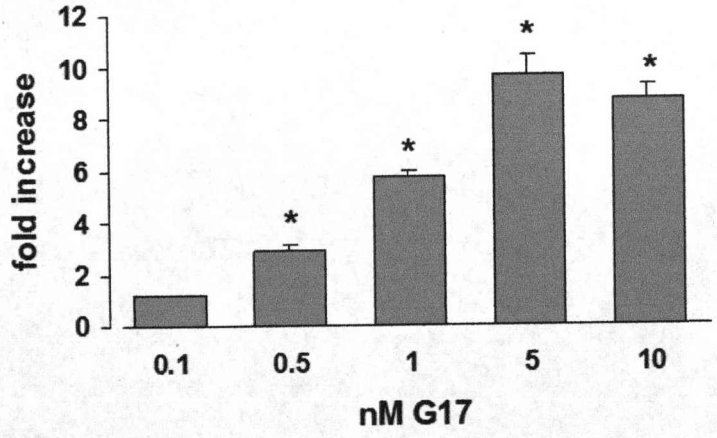


Fig 7: Stimulation of TIMP-3-luc increases progressively with stimulation from 0.5-5nM G17. 0.1nM G17 does not significantly increase expression (1.2 fold increase) and there was no difference in expression between 5 and 10nM G17 stimulation. Bars represent means of triplicates expressed as fold increase normalised to control ± SE. *$p<0.05$ ANOVA. (n=2).

5.3.5 Truncation of the TIMP-3 promoter to 1.35 Kb reduces activity in AGS-G_R cells

As I mentioned previously the luciferase-reporter construct used contained 1.6Kb of the TIMP-3 promoter, and in order to determine regions of the promoter required for responsiveness to G17 stimulation, sequential deletions of the promoter from 1.6-0.25Kb were generated.

Truncation of the promoter from 1.6Kb to 1.35Kb resulted in a reduction in activity with 5nM G17 stimulation. Truncations between 1.35Kb and 0.25Kb did not result in any further reduction in activity (Fig 8A).

Examination of the 1.6-1.35Kb region did not reveal any transcription factor binding sites of interest, however in the 0-0.25Kb region of the promoter there was a putative AP1 transcription factor binding site. Transcription factor binding sites were identified using TESS (http://www.cbil.upenn.edu/cgi-bin/tess).

Stimulation of AGS-G_R cells with 5nM G17 did not result in a change in empty pGL4 activity (Fig 8B).

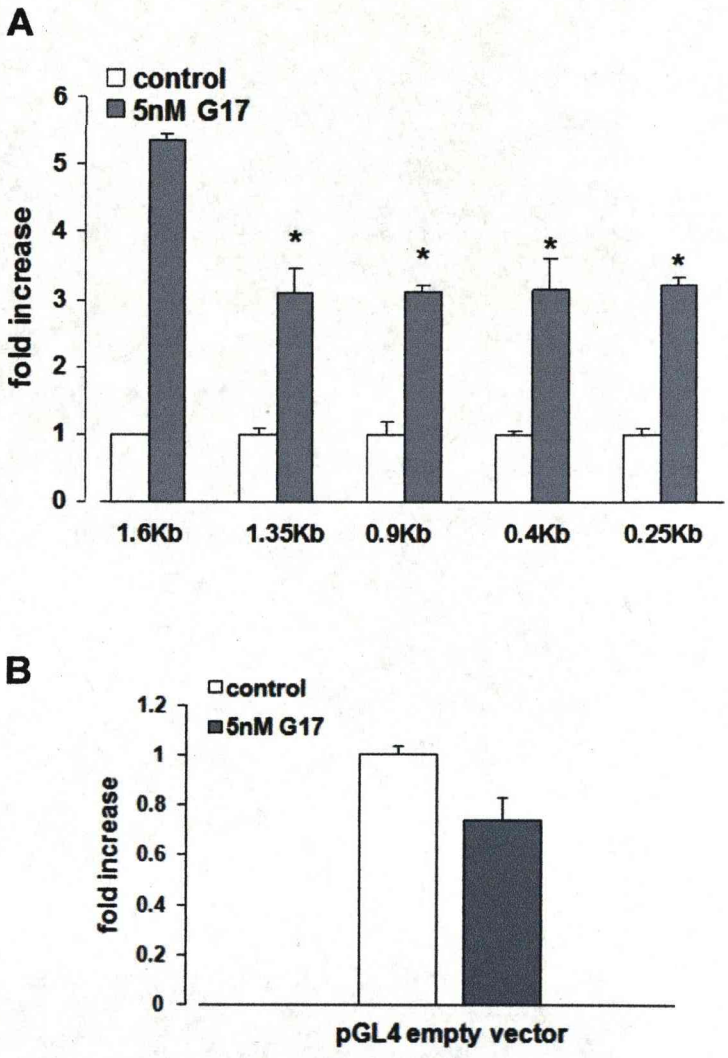


Fig 8: A. Truncation of the TIMP-3 promoter from 1.6-1.35Kb showing significant decrease in activity compared to 1.6Kb G17 stimulated *$p<0.05$ ANOVA. B. G17 stimulation caused no significant change in empty pGL4 activity. Normalised to control ± SE. *$p<0.05$ Student's t-test. One representative from 3 experiments is shown. Bars show means of triplicates expressed as fold increase.

5.3.6 MEK1/2 and PKC inhibitors reduce responses to gastrin stimulation of TIMP-3-luc

I had established that stimulation with G17 stimulates the expression of TIMP-3 in AGS-G_R cells (chapter 2 fig 6), and of TIMP-3-luc construct. To follow on from this I wanted to investigate the cellular mechanism of TIMP-3 stimulation by gastrin in AGS-G_R cells.

I found that UO-126, a MEK1/2 inhibitor markedly reduced the gastrin response in the large (1.6Kb) TIMP-3-luc construct and virtually abolished it in the 0.25Kb TIMP-3-luc construct. PKC inhibitor RO-320432 partially inhibited the effect of G17. However PI3K inhibitor LY-294002 had no effect on activity (Fig 9A). A similar pattern was seen using the TIMP-3-luc construct containing 0.25Kb of the TIMP-3 promoter, although RO-320432 had a greater effect on the response to G17 stimulation in this case construct (Fig 9B).

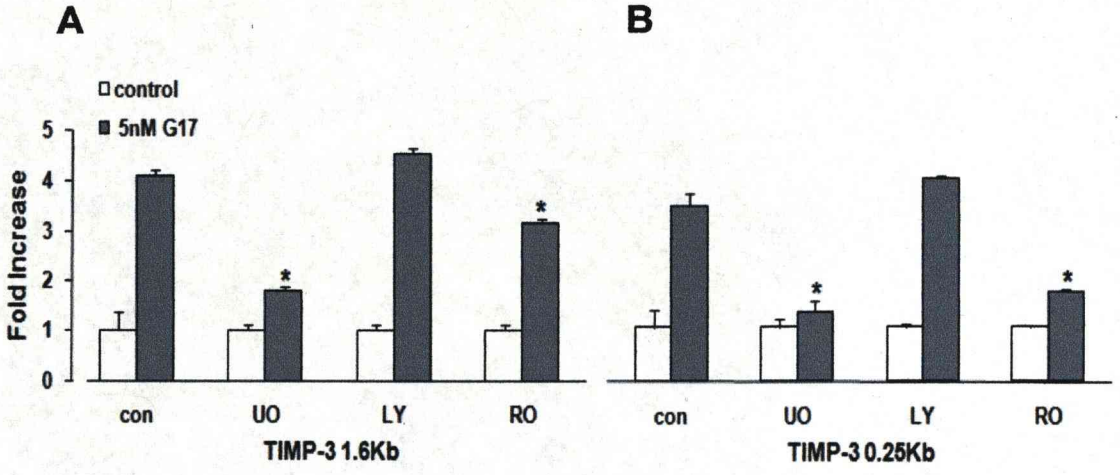


Fig 9: A. MEK1/2 inhibitor UO-126 markedly reduced the gastrin response in the 1.6Kb construct. PKC inhibitor RO-320432 partially inhibited the effect of G17. B. UO-126 virtually abolished the gastrin response in the 0.25Kb construct. One representative of at least 3 experiments is shown. Values are expressed as fold increase. Bars show means of triplicates ± SE. *$p<0.05$ ANOVA representing reduction in gastrin response compared to control G17 stimulated.

5.3.7 PMA stimulates expression of TIMP-3-luc in AGS-G_R cells

Since the PKC inhibitor RO-320432 partially inhibited the response of TIMP-3-luc to G17 stimulation, I decided to investigate whether PMA, an activator of PKC, stimulated TIMP-3-luc expression.

I found that PMA did significantly increase the activity of both 1.6Kb and 0.25Kb TIMP-3-luc in AGS-G_R cells compared to unstimulated cells. PMA did not, however, stimulate TIMP-3 expression to the same extent as stimulation with 5nM G17 (Fig 10 A&B), which is consistent with the data obtained using RO-32042.

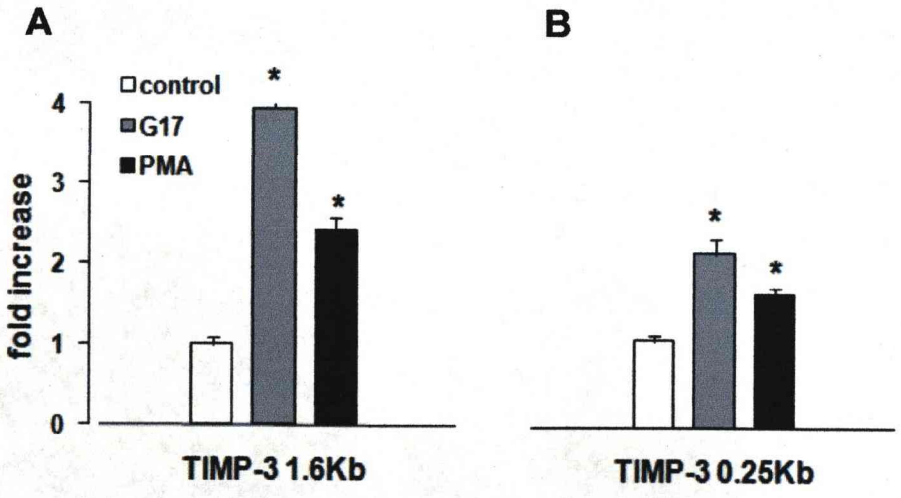


Fig 10: Stimulation with PMA (100nM) stimulated the activity of TIMP-3-luc 1.6Kb (A) and 0.25Kb (B) constructs. PMA did not stimulate activity to the same level as G17. One representative from 2-3 experiments shown. Bars show means of triplicates ± SE, expressed as fold increase normalised to control. *$p<0.05$ ANOVA.

5.3.8 Co-transfection with c-fos increases the expression of TIMP-3-luc compared to a control plasmid

I previously showed by Immunocytochemistry that G17 stimulation of AGS-G_R cells induces c-fos expression. This coupled with the fact that MEK inhibitor UO-126 markedly reduced the gastrin response in the 1.6Kb TIMP-3-luc construct and virtually abolished it in the 0.25Kb construct, suggested that stimulation of TIMP-3 expression by gastrin is regulated by AP1 family members.

Further evidence to support this was gained from the observation that co-transfection of AGS-G_R cells with TIMP-3-luc and c-fos resulted in increased luciferase activity of the the 1.6Kb and 0.25Kb constructs in otherwise unstimulated cells (Fig 11). The increase in activity was comparable to that seen with G17 stimulation (Fig 7). Co-transfection with TIMP-3 and c-jun had no effect on luciferase activity.

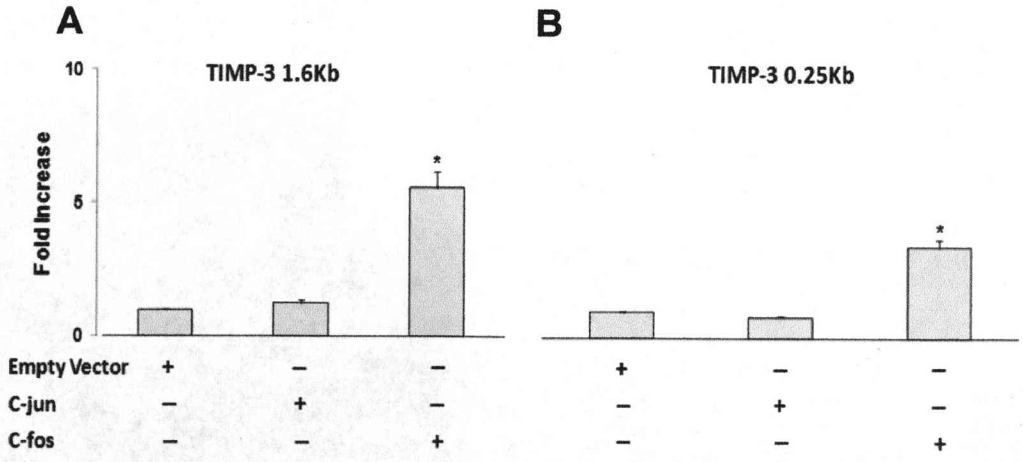


Fig 11: Co-transfection of AGS-G_R cells with TIMP-3-luc and c-fos resulted in increased luciferase activity. Representative of 3 experiments is shown. Bars represent means of triplicates ± SE, expressed as fold increase normalised to control (empty vector). Empty vector used was pCNA3. *$p<0.05$ ANOVA.

5.4 Discussion

TIMP-1 and -3 luciferase reporter constructs have been created in pGL4, are active in AGS-G_R cells, and luciferase expression is increased by G17. Truncations of the TIMP-3 promoter, inserted in pGL4 luciferase vector have been produced, whose expression is also increased by G17 stimulation of AGS-G_R cells.

Expression of luciferase from reporter constructs driven by TIMP-1, -3 and -4 promoters was increased by G17 stimulation of AGS-G_R cells. Transcription of the TIMP-3 promoter-reporter construct significantly increased in a dose-dependent manner from 0.1-10nM G17. Expression was increased significantly within 4-8h of stimulation with 1nM G17. Previous results from Western blot and Immunocytochemistry have shown that G17 stimulation increased the expression of TIMP-3 in AGS-G_R cells (chapter 2 Fig 6), although the expression of TIMP-1 and -4 were not affected. Because of previous data, the following experiments focused on TIMP-3, rather than TIMP-1 or -4.

Epidermal growth factor (EGF) also stimulated the expression of TIMP-1, -3 and -4 luciferase constructs in AGS-G_R cells. EGF can activate the MAPK pathway to induce the expression of many genes, including the *gastrin* gene (Chupreta *et al.* 2000). Gastrin can also induce the expression of members of the EGF family, leading to increased proliferation of gastric epithelial cells (Miyazaki *et al.* 1999, Wang *et al.* 2000).

Truncating the TIMP-3 promoter from 1.6Kb-1.35Kb caused a significant decrease in G17 induced stimulation of expression, however stimulation was not abolished. Truncating the promoter between positions 0.25Kb-1.35Kb did not further reduce expression. This suggests that a response element exists between 1.35-1.6Kb on the TIMP-3 promoter which mediates the stimulation of TIMP-3 expression by gastrin, however this is only partially responsible for inducing expression, as stimulation by gastrin was not completely abolished. A potential AP1 transcription factor binding site exists in the first 0.25Kb of the TIMP-3 promoter. It was not possible to use restriction enzyme digests to truncate the promoter in this region, but further work to mutate this site would be interesting, in order to investigate the importance of this sequence in the response of TIMP-3 expression to gastrin stimulation.

MEK1/2 inhibitor UO-126 significantly reduced, and PKC inhibitor RO-320432 partially abolished, the G17 induced stimulation of TIMP-3 expression. This suggests that gastrin stimulates TIMP-3 expression via activation of the MAPK pathway, and that PKC is responsible for some of the gastrin stimulated response.

It has been shown in previous work that The MAPK pathway is an important signalling route for the effects of PKC on the activity of AP-1, and this represents an important pathway for mediating the effects of gastrin on gene transcription (Davis *et al.* 1993). The MEK kinases activate extracellular signal-regulated kinases (ERKs) in response to various factors (Cobb *et al.* 1995), this upregulates the activity of many downstream targets, including transcription factors such as c-fos (Davis *et al.* 1993). In the pancreatic cell line AR4-2J, gastrin activates PKC, stimulates the MAPK

pathway, activates RhoA, and induces c-*fos*. (Dabrowski *et al.* 1997, Stepan *et al.* 1999).

It has been found that activation of the gastrin-CCK2 receptor stimulates cell migration via signalling mechanisms involving PKC and stimulation of EGF-R and the MAPK pathway (Noble *et al.* 2003)

Hocker *et al.* (1997) found that ERK-dependent signalling pathways mediated the stimulation of the HDC gene by gastrin. Regulation of VMAT2, Egr-1 and TFF1 by gastrin in AGS-G_R cells was also found to involve PKC and MAPK-dependent pathways (Watson *et al.* 2001, Raychowdhury *et al.* 2002, Khan *et al.* 2003). Gastrin-stimulated expression of the CCK2R also seems to be activated in part via PKC and MAPK dependent pathways (Ashurst *et al.* 2008).

Several studies have investigated the involvement of the MAPK pathway in the induction of TIMP expression. Activation of the MAPK pathway was found to play a key role in the stimulation of TIMP-1 expression and stimulation of proliferation of cancer cells, TIMP-3 expression in chondrocytes, TIMP-3 expression in Idiopathic pulmonary fibrosis, TIMP-1 expression in human fibrosarcoma cells and in human fibroblasts (Porter *et al.* 2004, Qureshi *et al.* 2005, Garcia-Alvarez *et al.* 2006, Kwak *et al.* 2006, Bodet *et al.* 2007).

The results presented here show that PMA did stimulate the expression of TIMP-3-luc, although stimulation was not to such a high level as that seen by G17. This concurs with PKC being partially responsible for activating TIMP-3 expression in response to gastrin stimulation.

Co-transfection of AGS-G_R cells with c-fos and TIMP-3-luc increased luciferase activity, which supports the view that gastrin stimulates TIMP-3 expression in AGS-G_R cells via activation of AP1. Further support for this comes from the fact that Immunocytochemistry showed that G17 stimulation of AGS-G_R cells induced c-fos expression (chapter 2 Fig 7).

Taken together the results from this chapter suggest that gastrin stimulates the MAPK pathway to increase TIMP-3 transcription via AP1, which results in inhibition of MMPs by TIMP-3, and contributes to remodelling of the ECM, which is important in a range of pathological conditions, such as inflammation (Fig 12).

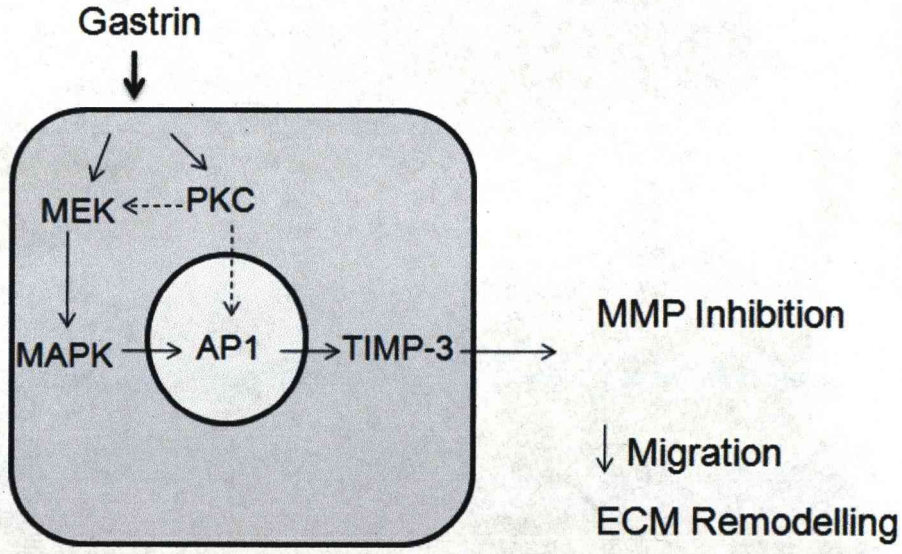


Fig 12: Representation of control of TIMP-3 expression by gastrin. Gastrin acts via MAPK pathway, activating AP1, resulting in TIMP-3 expression. This may involve activation by PKC. TIMP-3 has roles in MMP inhibition, and roles which may be independent of MMP inhibition, such as inhibition of migration. This has a role in ECM remodelling.

Chapter 6

Discussion

6.1 Major Findings

TIMP-1 and -3 mRNA expression increases in the corpus of Ins-GAS mice six months after infection with *Helicobacter felis*, compared to uninfected mice (Ch 3 Fig 3). In human corpus biopsies from patients with pathologically high circulating gastrin due to PA, the mRNA expression of all four TIMPs increased significantly, compared to patients with normal gastrin concentrations (Ch 3 Fig 4). When two PA patients were treated with octreotide, a somatostatin analogue, for 72h both patients showed a reduction in TIMP expression. Moreover after undergoing an antrectomy to permanently reduce the gastrin drive TIMP expression was reduced by almost 50% in two further patients (Ch 3 Fig 5).

Western blotting performed on whole cell lysates from cultured gastric myofibroblasts showed that all four TIMPs were expressed in patients with gastric adenocarcinoma (Ch 4 Fig 2), and TIMP-1, -2 and -4 expression was increased in myofibroblasts derived from the tumour, compared to adjacent normal tissue. TIMP-3 was expressed to a similar level in both sets of cells.

Stimulation of cultured gastric myofibroblasts, derived from normal gastric corpus mucosa, with HGF and TGF-β showed an increase in protein abundance of TIMP-1 in the medium. Stimulation with HGF, PDGF and TGF-β increased the abundance of TIMP-3 in whole cell lysates (Ch 4 Figs 4 & 5).

TIMP-1, -3 and -4 were expressed in the gastric cancer cell line AGS-G_R, and stimulation of these cells with G17 resulted in an increase in TIMP-3 expression, but did not alter TIMP-1 or -4 expression (Ch 4 Fig 6). Stimulation with 5nM G17 for 2h

also induced expression of the immediate early response gene c-fos in the nuclei of AGS-G_R cells (Ch 4 Fig 7).

Incubation of AGS-G_R cells with recombinant TIMP-3 inhibited gastrin-induced migration, and knocking down TIMP-3 expression with siRNA resulted in an increase in migration of gastrin stimulated AGS-G_R cells (Ch 4 Fig 8).

Stimulation with G17 increased the expression of a TIMP-3 luciferase-reporter construct in AGS-G_R cells. UO-126, a MEK1/2 inhibitor markedly reduced and PKC inhibitor RO-320432 partially inhibited the gastrin response of the TIMP-3-luc construct (Ch 5 Fig 9).

Co-transfection of AGS-G_R cells with TIMP-3-luc and c-fos resulted in increased luciferase activity to a comparable level to that seen with G17 stimulation (Ch 5 Fig 11).

6.2 Regulation of TIMP expression in the gastric mucosa of humans and a mouse model of hypergastrinaemia

Six months following *H. felis* infection, Ins-GAS mice showed increased expression of TIMP-1 and -3 mRNA in the gastric corpus, compared to non-infected control mice. Three months following *H. felis* infection there was no difference in TIMP expression. Preliminary data investigating TIMP expression with *H. pylori* infection in humans showed an increase in the expression of TIMP-1, -3 and -4, therefore the

results obtained in Ins-GAS mice are interesting as they correlate with those from human samples. In both humans and mice *Helicobacter* infection induces overexpression of TIMP-1 and -3, and the *Helicobacter* infected Ins-GAS mouse can therefore be used as an animal model for investigating TIMP expression in response to infection.

Ins-GAS mice have raised plasma gastrin concentrations and develop hyperplasia, similar to that seen in humans with CAG; older mice have an increased tendency to develop gastric cancer which is accelerated with *Helicobacter* infection (Wang *et al.* 1996, Wang *et al.* 2000). Gastrin levels in the Ins-GAS mouse samples used were similar in the control and infected mice. This suggests that in these experiments inflammation due to *H. felis* infection rather than hypergastrinaemia was responsible for the increase in TIMP expression.

Increased TIMP expression with *Helicobacter* infection may occur due to a corresponding increase in MMP expression. MMP expression is known to increase in response to inflammation associated with *H. pylori* infection. This contributes to ECM remodelling, which in some cases leads to gastric cancer (Wroblewski *et al.* 2003, Hemers *et al.* 2005, McCaig *et al.* 2006). TIMPs also have roles independent of MMP inhibition (Lambert *et al.* 2004), and this could also have important consequences in increased TIMP expression with *H. pylori* infection.

The expression of TIMP 1-4 mRNA increased significantly in gastric biopsy samples from patients with PA, compared to samples from patients with normal gastrin levels (<30pM). Patients with PA develop extremely high circulating gastrin levels, potentially rising to 2000pM, over a period of many years. I found no significant increase in TIMP expression between patients with normal gastrin levels and those

with elevated gastrin due to conditions other than PA, such as treatment with proton pump inhibitors (PPIs). PPIs are not likely to result in such high gastrin levels as those seen in PA, and this would occur over a shorter period of time, suggesting that very high gastrin levels, or that elevated gastrin over a very long period of time is required to increase TIMP expression.

In PA, ECL cell hyperplasia is common (Jensen 2002), in 5-10% of cases this progresses to the development of gastric carcinoid tumours (Bordi *et al.* 1991, Bordi *et al.* 1995). Large carcinoid tumours (>2cm) carry a significant risk of metastasis (Thomas *et al.* 1995). Antrectomy removes the gastrin secreting G-cells located in the antrum, and is often used as a treatment for ECL tumours. Octreotide is a somatostatin analogue and is known to decrease plasma gastrin levels and reduce ECL cell number in patients with CAG (Bordi *et al.* 1993, Ferraro *et al.* 1996). An octreotide suppression test, as described by Higham *et al.* (1998), can be used to determine whether large carcinoid tumours are responsive to gastrin, and thus whether antrectomy, rather than total gastrectomy, would be an appropriate treatment for large ECL carcinoid tumours. In samples from PA patients after octreotide infusion, and also pre and post antrectomy, TIMP overexpression due to hypergastrinaemia was reduced both after the octreotide suppression test and antrectomy. This suggests that upregulation of TIMP expression due to PA can be reversed after long term reduction of gastrin secretion by antrectomy.

6.3 The responsiveness of TIMP expression

Immunohistochemistry confirmed that TIMPs are expressed in a variety of cell types in *H. pylori* positive gastric mucosa. TIMP-1, -3 and -4 were expressed strongly in glandular epithelial cells, TIMP-3 and -4 were expressed in inflammatory cells, but TIMP-1 showed only weak positivity in these cells. TIMP-3 showed strong staining in myofibroblasts, while TIMP-1 showed weaker expression in myofibroblasts. TIMP-4 was localised exclusively in epithelial cells and not seen in myofibroblasts. This difference in cellular localisation between the individual TIMPs suggests varying roles for each TIMP in the gastric mucosa, and that the expression of each is influenced in a different way by *H. pylori* infection.

Western immunoblotting and Immunocytochemistry confirmed that TIMP-1 and -3 are expressed in cultured myofibroblasts from the site of a gastric adenocarcinoma and from adjacent macroscopically normal tissue. These can therefore be used to investigate TIMP expression and function in a sub-epithelial cell type.

Stimulation of myofibroblasts with HGF and TGF-β caused an increase in TIMP-1 abundance in the culture medium from these cells. Stimulation with HGF, PDGF and TGF-β stimulated TIMP-3 expression in whole cell lysates from myofibroblasts, showing that TIMP expression in myofibroblasts can be stimulated by growth factors. TGF-β can contribute to cancer progression by promoting tumour cell differentiation and metastasis (Moustakas *et al.* 2002), increased TIMP expression in myofibroblasts in response to TGF-β could be due to their roles in inhibition of ECM breakdown, TIMPs have also been found to promote metastasis in some tissues

(Baker *et al.* 2002). TGF-β is also involved in fibrosis, e.g in the kidney (Moustakas & Heldin 2007), and this could be a factor in TIMP upregulation in response to this growth factor. TGF-β induced TIMP-1 expression in cultured intestinal myofibroblasts and myometrial smooth muscle cells, and induced TIMP-3 expression in lung fibroblasts and human chondrocytes (McKaig *et al.* 2003, Qureshi *et al.* 2005, Garcia-Alvarez *et al.* 2006). TGF-β signalling in stromal myofibroblasts induces expression of HGF, and this promotes enhanced proliferation and invasion (Moustakas & Heldin 2007). PDGF has roles in promoting cell migration and survival (Tallquist *et al.* 2004). TIMPs have roles in inhibiting cell migration (Anand-Apte *et al.* 1996, Oh *et al.* 2004) and inhibiting apoptosis (Jiang *et al.* 2002), which could explain the increase in their expression in response to PDGF. PDGF is found to induce TIMP-1 expression in human dermal fibroblasts (Jinnin *et al.* 2005).

It is interesting that the expression of the different TIMPs varies in the gastric myofibroblasts. TIMP-1 was upregulated by growth factors in media from stimulated myofibroblasts while TIMP-3 was upregulated by growth factors in the cell lysates and not in medium. This could be due to the TIMPs having different roles and being regulated differently in this cell type. TIMP-3, unlike the other TIMPs, is tightly bound to the extracellular matrix and thus is not secreted like the other TIMPs (Woessner 2001), and this could explain why it was not seen in culture medium.

Epithelial-mesenchymal interactions are an important factor in the progression to cancer, and I went on to investigate TIMP expression in a gastric cancer cell line, AGS-G_R, which are stably transfected with the CCK2R, and respond to gastrin. TIMP-1, -3 and -4 are expressed in this cell type. TIMP-1 and -4 were expressed to similar level in unstimulated cells and in cells stimulated with G17; however TIMP-

3 expression was very low in unstimulated cells, but was induced by G17. This supports previous findings from our laboratory where gene array showed stimulation of TIMP-3 expression in AGS-G_R cells by gastrin (Varro *et al.* 2002b). These results showed that TIMP-3 expression in these cells is particularly interesting as it is the only TIMP influenced by gastrin.

Expression of luciferase from reporter constructs driven by TIMP-1, -3 and -4 promoters was increased by G17 stimulation of AGS-G_R cells. Transcription of the TIMP-3 promoter-reporter construct significantly increased in a dose-dependent manner from 0.1-10nM G17. Expression was increased significantly within 4-8h of stimulation with 1nM G17. Epidermal growth factor (EGF) also stimulated the expression of TIMP-1, -3 and -4 luciferase constructs in AGS-G_R cells. EGF can activate the MAPK pathway to induce the expression of many genes, including the *gastrin* gene (Chupreta *et al.* 2000). Gastrin can also induce the expression of members of the EGF family, leading to increased proliferation of gastric epithelial cells (Miyazaki *et al.* 1999, Wang *et al.* 2000).

Results from Western immunoblotting and Immunocytochemistry showed that G17 stimulation increased the expression of TIMP-3 in AGS-G_R cells, although the expression of TIMP-1 and -4 were not affected. G17 also stimulated the expression of a TIMP-3 luciferase construct in AGS-G_R cells. Also TIMP-3 mRNA expression in the gastric corpus was increased in *H. felis* infected Ins-GAS mice, and in patients with PA. In view of the results obtained at this stage, I decided to focus future experiments on TIMP-3 regulation and function in AGS-G_R cells.

Results from migration assays suggest that TIMP-3 inhibits the gastrin induced migration of AGS-G_R cells. It has been documented that gastrin stimulates the

migration of AGS-G_R cells (Varro *et al.* 2002b, Wroblewski *et al.* 2002), suggesting that elevated gastrin concentration plays an important role in the disruption of normal epithelial architecture. The inhibition of migration by TIMP-3 concurs with its MMP inhibitory function. MMP-7 and -9 are found to be upregulated in hypergastrinaemic conditions (Varro *et al.* 2007, Wroblewski *et al.* 2002), and the increased expression of TIMP-3 seen could be in response to increased MMP expression. Elevated TIMP -3 expression could then act by inhibiting breakdown of the ECM by the MMPs, thus inhibiting migration.

6.4 The cellular mechanism of TIMP expression

Expression of luciferase from a reporter construct driven by the TIMP-3 promoter is increased by G17 stimulation of AGS-G_R cells. Truncating the TIMP-3 promoter from 1.6Kb-1.35Kb caused a significant decrease in G17 induced stimulation of expression, however stimulation was not abolished. This suggests a response element exists in this region of the TIMP-3 promoter, which mediates the gastrin stimulation of TIMP-3 expression. Stimulation by gastrin was not completely abolished and this indicates that this response element is only partially responsible for inducing expression. A potential AP1 transcription factor binding site was located in the first 0.25Kb of the TIMP-3 promoter.

A MEK1/2 inhibitor significantly reduced, and a PKC inhibitor partially abolished, the G17 induced stimulation of TIMP-3 expression. This suggests that gastrin stimulates TIMP-3 expression via activation of the MAPK pathway, and that PKC is responsible for some of the gastrin stimulated response.

Previous work has shown that the MAPK pathway is an important signalling route for the effects of PKC on the activity of AP-1, and this represents an important pathway for mediating the effects of gastrin on gene transcription (Davis *et al.* 1993). Activation of the CCK2R stimulates cell migration via signalling mechanisms involving PKC and stimulation of EGF-R and the MAPK pathway (Noble *et al.* 2003). Regulation of VMAT2, Egr-1 and TFF1 by gastrin in AGS-G_R cells was also found to involve PKC and MAPK-dependent pathways (Watson *et al.* 2001, Raychowdhury *et al.* 2002, Khan *et al.* 2003). Gastrin stimulated expression of the CCK2R also seems to be activated in part via PKC and MAPK dependent pathways (Ashurst *et al.* 2008).

Activation of the MAPK pathway was found to play a key role in the stimulation of TIMP-1 expression in human fibrosarcoma cells and in human fibroblasts, and also TIMP-3 expression in chondrocytes and in idiopathic pulmonary fibrosis (Porter *et al.* 2004, Qureshi *et al.* 2005, Garcia-Alvarez *et al.* 2006, Kwak *et al.* 2006, Bodet *et al.* 2007).

PMA was shown to stimulate the expression of the TIMP-3 luciferase reporter construct, although not to the same level as that seen by G17 stimulation. This agrees with PKC being partially responsible for activating TIMP-3 expression in response to gastrin stimulation.

My results showed that gastrin stimulates the expression of c-fos in AGS-G_R cells. *c-fos* is part of the *Fos* family of genes, which in combination with the Jun families, forms the AP-1 immediate early response transcription factor complex (Karamouzis *et al.* 2007). The AP-1 proteins are induced in response to a broad spectrum of stimuli including cytokines, growth factors, oncogenes and stress signals (Young *et al.* 2006), and play a central role in the control of complex biological processes like cell proliferation and differentiation (Angel *et al.* 1991). AP-1 can be regulated via the MAPK pathway (Hurd *et al.* 2002).

Previous studies in our laboratory have shown that gastrin induces the expression of MMP-9 (Wroblewski *et al.* 2002) and PAI-2 (Varro *et al.* 2002b) via AP-1 activation. My results suggest TIMP-3 may be induced by gastrin in a similar manner. AP-1 has been shown to be essential for the induction of TIMP-1 gene expression by TGF-β1 (Hall *et al.* 2003), to be involved in the induction of TIMP-1 and -3 in human fibroblasts (Edwards *et al.* 1996), and TIMP-1 and -2 expression is regulated by AP-1 in breast cancer cell lines (Bachmeier *et al.* 2005). In the pancreatic cell line AR4-2J, gastrin activates PKC, stimulates the MAPK pathway, activates RhoA, and induces c-*fos*. (Dabrowski *et al.* 1997, Stepan *et al.* 1999).

Co-transfection of AGS-G_R cells with c-fos and TIMP-3-luc increased luciferase activity, which, in addition to the finding that G17 stimulation of AGS-G_R cells induced c-fos expression, supports the view that gastrin stimulates the MAPK pathway to increase TIMP-3 transcription via AP1, which results in inhibition of MMPs by TIMP-3, and contributes to remodelling of the ECM.

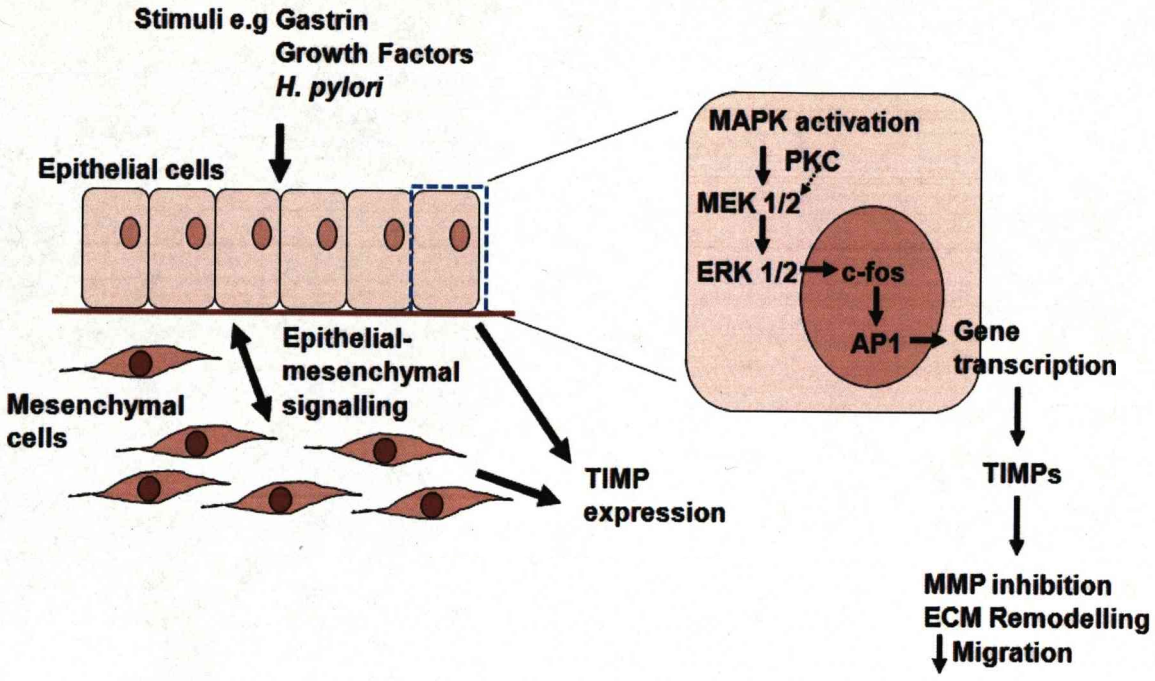


Fig 1: Summary of findings. Stimuli such as gastrin, growth factors and *H. pylori* infection stimulate epithelial cells. Epithelial and mesenchymal cells communicate with each other via epithelial-mesenchymal signalling. Expression of TIMPs is upregulated by stimuli in epithelial and mesenchymal cells e.g. myofibroblasts. Signalling e.g. by gastrin activates the MAPK pathways, PKC being partially responsible for this activation. Expression of c-fos is stimulated in the nucleus, which forms part of the AP1 transcription factor complex, this leads to gene transcription, including TIMPs. TIMPs inhibit MMP activity and migration which contribute to ECM remodelling.

6.5 Future work

- A potential AP1 transcription factor binding site exists in the first 0.25Kb of the TIMP-3 promoter used to drive luciferase expression in reporter constructs. It was not possible to use restriction enzyme digests to truncate the promoter in this region, but further work to mutate this site would be interesting, in order to investigate the importance of this sequence in the response of TIMP-3 expression to gastrin stimulation.

- Expression of luciferase activity by a reporter construct driven by a TIMP-3 promoter has been used to investigate the response of TIMP-3 to gastrin in AGS-G_R cells. TIMP-1 and -4 are also expressed by this cell line, and luciferase promoter constructs driven by these promoters exist. Initial results showed that the expression of these constructs was stimulated by G17 in AGS-G_R cells. Further investigation into the regulation of TIMP-1 and -4 in response to gastrin would be interesting.

- It was found that TIMP-3 inhibited gastrin induced migration of AGS-G_R cells, futher investigation into the function of TIMP-3 in AGS-G_R cells would be interesting, for example by performing invasion or proliferation assays.

Chapter 7

References

References

Ahmed A, Smoot D, Littleton G, Tackey R, Walters CS, Kashanchi F, Allen CR, Ashktorab H. *Helicobacter pylori* inhibits gastric cell cycle progression. *Microbes Infect.* (2000) **2** 1159–1169

Akagi M, Kawaguchi M, Liu W, McCarty MF, Takeda A, Fan F, Stoeltzing O, Parikh AA, Jung YD, Bucana CD, Mansfield PF, Hicklin DJ, Ellis LM. Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. *Br J Cancer* (2003) **88** 796-802

Akhurst RJ, Balmain A. Genetic events and the role of TGF beta in epithelial tumour progression. *J Pathol.* (1999) **187** (1) 82-90

Alman BA, Naber SP, Terek RM, Jiranek WA, Goldberg MJ, Wolfe HJ. Platelet-derived growth factor in fibrous musculoskeletal disorders: a study of pathologic tissue sections and in vitro primary cell cultures. *J Orthop Res.* (1995) **13** (1) 67-77

Anan K, Morisaki T, Katano M, Ikubo A, Kitsuki H, Uchiyama A, Kuroki S, Tanaka M, Torisu M. Vascular endothelial growth factor and platelet-derived growth factor are potential angiogenic and metastatic factors in human breast cancer. *Surgery.* (1996) **119** (3) 333-9

Anand-Apte B, Bao L, Smith R, Iwata K, Olsen BR, Zetter B and Apte SS. A review of tissue inhibitor of metalloproteinases-3 (TIMP-3) and experimental analysis of its effect on primary tumour growth. *Biochem Cell Biol* (1996) **74** 853-62

Angel P and Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer.* (1991) **1072** (2-3) 129-157

Antoniades HN, Scher CD, Stiles CD. Purification of human platelet-derived growth factor. *Proc Natl Acad Sci U S A.* (1979) **76** (4) 1809-13

Apte SM, Bucana CD, Killion JJ, Gershenson DM, Fidler IJ. Expression of platelet-derived growth factor and activated receptor in clinical specimens of epithelial ovarian cancer and ovarian carcinoma cell lines. *Gynecol Oncol.* (2004) **93** (1) 78-86

Ashworth JL, Murphy G, Rock MJ, Sherratt MJ, Shapiro SD, et al. Fibrillin degradation by matrix metalloproteinases: implications for connective tissue remodelling. *Biochem. J.* (1999) **340** 171– 81

Bachmeier BE, Vené R, Iancu CM, Pfeffer U, Mayer B, Noonan D, Albini A, Jochum M, Nerlich AG. Transcriptional control of cell density dependent regulation of matrix metalloproteinase and TIMP expression in breast cancer cell lines. *Thromb Haemost.* (2005) **93** (4) 761-9

Baker AH, Zaltsman AB, George SJ, Newby AC. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis.

J Clin Invest. (1998) **101** (6) 1478-87

Baker EA, Leaper DJ, Hayter JP, Dickenson AJ. The matrix metalloproteinase system in oral squamous cell carcinoma. *Br J Oral Maxillofac Surg.* (2006) **44** (6) 482-6

Basset P, Bellocq JP, Wolf C, et al: A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature* (1990) **348** 699-704

Battegay EJ, Rupp J, Iruela-Arispe L, Sage EH and Pech M. PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF -receptors. *J. Cell Biol.* (1994) **125** 917-928

Bauer E A, Cooper T W, Huang J S, Altman J, and Deuel T F. Stimulation of in vitro human skin collagenase expression by platelet-derived growth factor. *Proc Natl Acad Sci USA* (1985) **82** 4132-4136

Bayliss WM and Starling EH. The mechanism of pancreatic secretion. *Journal of Physiology.* (1902) **28** 325-353.

Beaumont W. Gastric juice and the physiology of digestion. Plattsburgh, FP Allen (1833)

Bebb JR, Letley DP, Thomas RJ, Aviles F, Collins HM, Watson SA, Hand NM, Zaitoun A, Atherton JC. Helicobacter pylori upregulates matrilysin (MMP-7) in epithelial cells in vivo and in vitro in a Cag dependent manner. *Gut.* (2003) **52** (10) 1408-13

Bello L, Lucini V, Carrabba G, Giussani C, Machluf M, Pluderi M, Nikas D, Zhang J, Tomei G, Villani RM, Carroll RS, Bikfalvi A, Black PM. Simultaneous inhibition of glioma angiogenesis, cell proliferation, and invasion by a naturally occurring fragment of human metalloproteinase-2. *Cancer Res.* (2001) **61** (24) 8730-6

Bergin PJ, Anders E, Sicheng W, Johnsson E , Andersson J, Lonroth H, Michetti P, Pan-Hammarstrom Q and Quiding-Jarbrink M. Increased proliferation of matrix metalloproteinases in Helicobacter pylori associated human gastritis. *Helicobacter* (2004) **9** (3) 201-210.

Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin CH, Alitalo K, Eriksson U. PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. *Nat Cell Biol.* (2001) **3** (5) 512-6

Bertaux B, Hornebeck W, Eisen AZ, Dubertret L. Growth stimulation of human keratinocytes by tissue inhibitor of metalloproteinases. *J Invest Dermatol.* (1991) **97** (4) 679-85

Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell.* (2001) **12** (1) 27-36

Bhowmick NA, Neilson EG and Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature*. (2004) **432** 332-337

Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol*. (2003) **4** (12) 915-25

Bladt F, Riethmacher D, Isenmann S, Aguzzi A & Birchmeier C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* (1995) **376** 768–771

Blatti SP, Foster DN, Ranganathan G, Moses HL, Getz MJ. Induction of fibronectin gene transcription and mRNA is a primary response to growth-factor stimulation of AKR-2B cells. *Proc Natl Acad Sci U S A*. (1988) **85** (4) 1119-23

Blenis J, Hawkes SP. Characterization of a transformation-sensitive protein in the extracellular matrix of chicken embryo fibroblasts. *J Biol Chem*. (1984) **259** (18) 11563-70

Bodet C, Andrian E, Tanabe S, Grenier D. Actinobacillus actinomycetemcomitans lipopolysaccharide regulates matrix metalloproteinase, tissue inhibitors of matrix metalloproteinase, and plasminogen activator production by human gingival fibroblasts: a potential role in connective tissue destruction. *J Cell Physiol*. (2007) **212** (1) 189-94

Bodger K, Ahmed S, Pazmany L, Pritchard DM, Michael A, Khan AL, Dimaline R, Dockray GJ, Varro A. Altered gastric corpus expression of tissue inhibitors of metalloproteinases in human and murine Helicobacter infection. *J Clin Pathol*. (2008) **61** (1) 72-8.

Bordi C, D'Adda T, Azzoni C, Pilato FP, Caruana P. Hypergastrinemia and gastric enterochromaffin-like cells. *Am J Surg Pathol*. (1995) **19** Suppl 1 S8-19.

Bordi C, Azzoni C, Pilato FP, Robutti F, D'Ambra G, Caruana P, Rindi G, Corleto VD, Annibale B, Delle Fave G. Morphometry of gastric endocrine cells in hypergastrinemic patients treated with the somatostatin analogue octreotide. *Regul Pept*. (1993) **47** (3) 307-18.

Bordi C, Yu JY, Baggi MT, Davoli C, Pilato FP, Baruzzi G, Gardini G, Zamboni G, Franzin G, Papotti M, et al. Gastric carcinoids and their precursor lesions. A histologic and immunohistochemical study of 23 cases. *Cancer*. (1991) **67** (3) 663-72.

Boström H, Willetts K, Pekny M, Levéen P, Lindahl P, Hedstrand H, Pekna M, Hellström M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Törnell J, Heath JK, Betsholtz C. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell*. (1996) **85** (6) 863-73

Bramhall SR, Rosemurgy A, Brown PD, Bowry C, Buckels JA; Marimastat Pancreatic Cancer Study Group. Marimastat as first-line therapy for patients with

unresectable pancreatic cancer: a randomized trial. *J Clin Oncol.* (2001) **19** (15) 3447-55

Brand K, Baker AH, Perez-Cantó A, Possling A, Sacharjat M, Geheeb M, Arnold W. Treatment of colorectal liver metastases by adenoviral transfer of tissue inhibitor of metalloproteinases-2 into the liver tissue. *Cancer Res.* (2000) **60** (20) 5723-30

Brittan M, Wright NA. Gastrointestinal stem cells. *J Pathol.* (2002) **197** (4) 492-509

Brown PD. Ongoing trials with matrix metalloproteinase inhibitors. *Expert Opin Investig Drugs.* (2000) **9** (9) 2167-77

Bryckaert MC, Rendu F, Tobelem G and Wasteson A. Collagen-induced binding to human platelets of platelet-derived growth factor leading to inhibition of P43 and P20 phosphorylation. *J. Biol Chem* (1989) **264** 4336-4341

Calam J, Gibbons A, Healey ZV, Bliss P, Arebi N. How does *Helicobacter pylori* cause mucosal damage? Its effect on acid and gastrin physiology. *Gastroenterology.* (1997) **113** (6 Suppl) S43-9

Canalis E. Effect of platelet-derived growth factor on DNA and protein synthesis in cultured rat calvaria. *Metabolism* (1981) **30** 970-975

Carvalho I, Milanezi F, Martins A, Reis RM, Schmitt F. Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. *Breast Cancer Res.* (2005) **7** (5) R788-95

Castagnino P, Soriano JV, Montesano R and Bottaro DP. Induction of tissue inhibitor of metalloproteinases-3 is a delayed early cellular response to hepatocyte growth factor. *Oncogene.* (1998) **17** 481-492

Caterina NC, Windsor LJ, Yermovsky AE, Bodden MK, Taylor KB, Birkedal-Hansen H, Engler JA. Replacement of conserved cysteines in human tissue inhibitor of metalloproteinases-1. *J Biol Chem.* (1997) **272** (51) 32141-9

Chang L and Karin M. Mammalian MAP kinase signalling cascades. *Nature* (2001) **410** 37-40

Chesler L, Golde DW, Bersch N, Johnson MD. Metalloproteinase inhibition and erythroid potentiation are independent activities of tissue inhibitor of Metalloproteinases-1. *Blood.* (1995) **86** (12) 4506-15

Chirco R, Liu XW, Jung KK, Kim HR. Novel functions of TIMPs in cell signaling. *Cancer Metastasis Rev.* (2006) **25** (1) 99-113

Chung CK and Antoniades HN. Expression of c-*sis*/platelet-derived growth factor B, insulin-like growth factor I, and transforming growth factor a messenger RNAs and their respective receptor messenger RNAs in primary human gastric carcinomas: in vivo studies with in situ hybridization and immunocytochemistry. *Cancer Res.* (1992) **52** 3453-3459

Chupreta S, Du M, Todisco A, Merchant JL. EGF stimulates gastrin promoter through activation of Sp1 kinase activity. *Am J Physiol Cell Physiol.* (2000) **278** (4) C697-708

Claesson-Welsh L. Platelet-derived growth factor receptor signals. *J Biol Chem.* (1994) **269** (51) 32023-6.

Cobb MH, Goldsmith EJ. How MAP kinases are regulated. *J Biol Chem.* (1995) **270** 14843-6.

Cohen S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J. Biol. Chem.* (1962) **237** 1555–1562

Correa P, Chen VW. Gastric cancer. *Cancer Surv.* (1994) **19-20** 55-76

Correa P, Fontham ET, Bravo JC, Bravo LE, Ruiz B, Zarama G, Realpe JL, Malcom GT, Li D, Johnson WD, Mera R. Chemoprevention of gastric dysplasia: randomized trial of antioxidant supplements and anti-helicobacter pylori therapy. *J Natl Cancer Inst.* (2000) **92** (23) 1881-8

Coussens LM, Shapiro SD, Soloway PD, Werb Z. Models for gain-of-function and loss-of-function of MMPs. Transgenic and gene targeted mice. *Methods Mol Biol.* (2001) **151** 149-79

Crews C, Alessandrini A, Erikson R. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* (1992) **258** 478–480

Curci JA, Liao S, Huffman MD, Shapiro SD, Thompson RW. Expression and localization of macrophage elastase (matrix metalloproteinase-12) in abdominal aortic aneurysms. *J. Clin. Invest.* (1998) **102** 1900– 10

Curran S, Murray GI. Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. *Eur J Cancer.* (2000) **36** (13 Spec No) 1621-30

Dabrowski A, Detjen KM, Logsdon CD, Williams JA. Stimulation of both CCK-A and CCK-B receptors activates MAP kinases in AR42J and receptor-transfected CHO cells. (1997) *Digestion* **58** 361– 67

Daub H, Wallasch C, Lankenau A, Herrlich A, Ullrich A. Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.* (1997) **16** (23) 7032-44

Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem.* (1993) **268** (20) 14553-6.

de Visser KE, Kast WM. Effects of TGF-beta on the immune system: implications for cancer immunotherapy. *Leukemia.* (1999) **13** (8) 1188-99

Del Carlo M, Cole AA, Levine LA. Differential calcium independent regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by interleukin-1beta and transforming growth factor-beta in Peyronie's plaque fibroblasts. *J Urol.* (2008) **179** (6) 2447-55

Di Renzo MF, Olivero M, Ferro S, Prat M, Bongarzone I, Pilotti S, Belfiore A, Costantino A, Vigneri R, Pierotti MA, et al. Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. *Oncogene.* (1992) **7** (12) 2549-53

Di Renzo MF, Olivero M, Giacomini A, Porte H, Chastre E, Mirossay L, Nordlinger B, Bretti S, Bottardi S, Giordano S, et al. Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. *Clin Cancer Res.* (1995) **1** (2) 147-54

Díaz R, Kim JW, Hui JJ, Li Z, Swain GP, Fong KSK, Csiszar K, Russo PA, Rand EB, Furth EE and Wells RG. Evidence for the epithelial to mesenchymal transition in biliary atresia fibrosis. *Human Pathology.* (2007) Sep 27 E-pub ahead of print.

Dimaline R, Sandvik AK, Evans D, Forster ER, Dockray GJ. Food stimulation of histidine decarboxylase messenger RNA abundance in rat gastric fundus. *J Physiol.* (1993) **465** 449-58.

Dimaline R, Evans D, Forster ER, Sandvik AK, Dockray GJ. Control of gastric corpus chromogranin A messenger RNA abundance in the rat. *Am J Physiol.* (1993) **264** (3 Pt 1) G583-8.

Dimaline R, Struthers J. Expression and regulation of a vesicular monoamine transporter in rat stomach: a putative histamine transporter. *J Physiol.* (1996) **490** (Pt 1) 249-56.

Dockray GJ, Varro A, Dimaline R, Wang T. The gastrins: their production and biological activities. *Annu Rev Physiol.* (2001) **63** 119-39

Dockray G, Dimaline R, Varro A. Gastrin: old hormone, new functions. *Pflugers Arch.* (2005) **449** (4) 344-55

Ebert M, Yokoyama M, Friess H, Kobrin MS, Büchler MW, Korc M. Induction of platelet-derived growth factor A and B chains and over-expression of their receptors in human pancreatic cancer. *Int J Cancer.* (1995) **62** (5) 529-35

Edkins JS. On the chemical mechanism of gastric secretion. *Proceedings of the Royal Society Series B.* (1905) **76** 376.

Edwards DR, Leco KJ, Beaudry PP, Atadja PW, Veillette C, Riabowol KT. Differential effects of transforming growth factor-beta 1 on the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in young and old human fibroblasts. *Exp Gerontol.* (1996) **31** (1-2) 207-23

English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, Cobb MH. New insights into the control of MAP kinase pathways. *Exp Cell Res.* (1999) **253** (1) 255-70

Fan X, Crowe SE, Behar S, Gunasena H, Ye G, Haeberle H, Van Houten N, Gourley WK, Ernst PB, Reyes VE. The effect of class II major histocompatibility complex expression on adherence of *Helicobacter pylori* and induction of apoptosis in gastric epithelial cells: a mechanism for T helper cell type 1-mediated damage. *J Exp Med* (1998) **187** 1659–1669

Fan X, Gunasena H, Cheng Z, Espejo R, Crowe SE, Ernst PB, Reyes VE. *Helicobacter pylori* urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. *J. Immunol.* (2000) **165** 1918–1924

Felbor U, Stöhr H, Amann T, Schönherr U, Weber BH. A novel Ser156Cys mutation in the tissue inhibitor of metalloproteinases-3 (TIMP3) in Sorsby's fundus dystrophy with unusual clinical features. *Hum Mol Genet.* (1995) **4** (12) 2415-6

Ferraro G, Annibale B, Marignani M, Azzoni C, D'Adda T, D'Ambra G, Bordi C, delle Fave G. Effectiveness of octreotide in controlling fasting hypergastrinemia and related enterochromaffin-like cell growth. *J Clin Endocrinol Metab.* (1996) **81** (2) 677-83

Forman D, Newell DG, Fullerton F, Yarnell JW, Stacey AR, Wald N, Sitas F. Association between infection with Helicobacter pylori and risk of gastric cancer: evidence from a prospective investigation. *BMJ.* (1991) **302** (6788) 1302-5

Fox JG, Wang TC. Helicobacter pylori--not a good bug after all! *N Engl J Med.* (2001) **345** (11) 829-32

Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev.* (2004) **15** (4) 197-204

Fu, S. *et al.* Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. *Gastroenterology* (1999) **116** 1319–1329

Gallagher PG, Bao Y, Prorock A, Zigrino P, Nischt R, Politi V, Mauch C, Dragulev B and Fox JW. Gene expression profiling reveals cross-talk between melanoma and fibroblasts: Implications for host-tumour interactions in metastasis. *Cancer Res.* (2005) **65** 4134-4146.

Garcia-Alvarez J, Ramirez R, Checa M, Nuttall RK, Sampieri CL, Edwards DR, Seiman M and Pardo A. Tissue inhibitor of metalloproteinase-3 is upregulated by transforming growth factor-beta1 in vitro and expressed in fibroblastic foci in idiopathic pulmonary fibrosis. *Exp Lung Res* (2006) **32** 201-214

Gasson JC, Golde DW, Kaufman SE, Westbrook CA, Hewick RM, Kaufman RJ, Wong GG, Temple PA, Leary AC, Brown EL, et al. Molecular characterization and

expression of the gene encoding human erythroid-potentiating activity. *Nature.* (1985) **315** (6022) 768-71

Gibril F, Schumann M, Pace A, Jensen RT. Multiple endocrine neoplasia type 1 and Zollinger-Ellison syndrome: a prospective study of 107 cases and comparison with 1009 cases from the literature. *Medicine (Baltimore).* (2004) **83** (1) 43-83

Gill SE, Pape MC, Khokha R, Watson AJ, Leco KJ. A null mutation for tissue inhibitor of metalloproteinases-3 (Timp-3) impairs murine bronchiole branching morphogenesis. *Dev Biol.* (2003) **261** (2) 313-23

Gill SE, Pape MC, Leco KJ. Tissue inhibitor of metalloproteinases 3 regulates extracellular matrix--cell signaling during bronchiole branching morphogenesis. *Dev Biol.* (2006) **298** (2) 540-54

Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: A model of glioblastoma multiforme pathophysiology. *Mol Biol Cell* (1993) **4** 121-133

Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol.* (1997) **74** (2) 111-22

Göõz M, Göõz P, Smolka AJ. Epithelial and bacterial metalloproteinases and their inhibitors in H. pylori infection of human gastric cells. *Am J Physiol Gastrointest Liver Physiol.* (2001) **281** (3) G823-32

Görögh T, Beier UH, Bäumken J, Meyer JE, Hoffmann M, Gottschlich S, Maune S. Metalloproteinases and their inhibitors: influence on tumor invasiveness and metastasis formation in head and neck squamous cell carcinomas. *Head Neck.* (2006) **28** (1) 31-9

Gregory RA and Tracy HJ. The constitution and properties of two gastrins extracted from hog antral mucosa. *Gut.* (1964) **5** 103-114.

Guedez L, Stetler-Stevenson WG, Wolff L, Wang J, Fukushima P, Mansoor A, Stetler-Stevenson M. In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. *J Clin Invest.* (1998) **102** (11) 2002-10

Hagemann C, Rapp UR. Isotype-specific functions of Raf kinases. *Exp Cell Res* (1999) **253** 34–46

Hall MC, Young DA, Waters JG, Rowan AD, Chantry A, Edwards DR, Clark IM. The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1. *J Biol Chem.* (2003) **278** (12) 10304-13

Harris RC, Chung E, and Coffey RJ. EGF receptor ligands. *Exp Cell Res.* (2003) **284** (1) 2–13

Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci.* (1994) **107** (Pt 9) 2373-9

Hazzalin CA, Mahadevan LC. MAPK-regulated transcription: a continuously variable gene switch? *Nat Rev Mol Cell Biol.* (2002) **3** (1) 30-40

Heldin CH, Wasteson A, Westermark B. Platelet-derived growth factor. *Mol Cell Endocrinol.* (1985) **39** (3) 169-87

Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature.* (1997) **390** (6659) 465-71

Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev.* (1999) **79** (4) 1283-316

Hemers E, Duval C, McCaig C, Handley M, Dockray GJ and Varro A. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: Implications for epithelial-mesenchymal signalling. *Cancer Res.* (2005) **65** 7363-7369

Hersey SJ, Sachs G. Gastric acid secretion. *Physiol Rev.* (1995) **75** (1) 155-89

Higham AD, Bishop LA, Dimaline R, Blackmore CG, Dobbins AC, Varro A, Thompson DG, Dockray GJ. Mutations of Reglalpha are associated with enterochromaffin-like cell tumor development in patients with hypergastrinemia. *Gastroenterology.* (1999) **116** (6) 1310-8

Higham AD, Dimaline R, Varro A, Attwood S, Armstrong G, Dockray GJ, Thompson DG. Octreotide suppression test predicts beneficial outcome from antrectomy in a patient with gastric carcinoid tumor. *Gastroenterology.* (1998) **114** (4) 817-22

Hinz B, Phan SH, Thannickal VJ, Gall A, Bochaton-Piallat ML and Gabbiani G. The myofibroblast. One function, multiple origins. *Am J Path.* (2007) **170** 1807-1816

Hirschowitz BI, Griffith J, Pellegrin D, Cummings OW. Rapid regression of enterochromaffinlike cell gastric carcinoids in pernicious anemia after antrectomy. *Gastroenterology.* (1992) **102** (4 Pt 1) 1409-18

Höcker M, Henihan RJ, Rosewicz S, Riecken EO, Zhang Z, Koh TJ, Wang TC. Gastrin and phorbol 12-myristate 13-acetate regulate the human histidine decarboxylase promoter through Raf-dependent activation of extracellular signal-regulated kinase-related signaling pathways in gastric cancer cells. *J Biol Chem.* (1997) **272** (43) 27015-24

Honda S, Fujioka T, Tokieda M, Satoh R, Nishizono A, Nasu M. Development of Helicobacter pylori-induced gastric carcinoma in Mongolian gerbils. *Cancer Res.* (1998) **58** (19) 4255-9

Hurd TW, Culbert AA, Webster KJ, et al. Dual role for mitogen-activated protein kinase (Erk) in insulin-dependent regulation of Fra1 (fos-related antigen-1) transcription and phosphorylation. *Biochem J* (2002) **368** 573–80

Isaka Y, Fujiwara Y, Ueda N, Kaneda Y, Kamada T, and. Imai E. Glomerulosclerosis induced by in vivo transfection of transforming growth factor-β or platelet-derived growth factor gene into the rat kidney. *J Clin Invest.* (1993) **92** 2597-2601

Işlekel H, Oktay G, Terzi C, Canda AE, Füzün M, Küpelioğlu A. Matrix metalloproteinase-9,-3 and tissue inhibitor of matrix metalloproteinase-1 in colorectal cancer: relationship to clinicopathological variables. *Cell Biochem Funct.* (2007) **25** (4) 433-41

Itoh T, Tanioka M, Yoshida H, et al: Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res* (1998) **58** 1048-1051

Itoh Y, Seiki M. MT1-MMP: a potent modifier of pericellular microenvironment. *J Cell Physiol.* (2006) **206** (1) 1-8

Jaworski DM, Soloway P, Caterina J, Falls WA. Tissue inhibitor of metalloproteinase-2(TIMP-2)-deficient mice display motor deficits. *J Neurobiol.* (2006) **66** (1) 82-94

Jensen RT. Involvement of cholecystokinin/gastrin-related peptides and their receptors in clinical gastrointestinal disorders. *Pharmacol Toxicol.* (2002) **91** (6) 333-50.

Jiang Y, Goldberg ID and Shi YE. Complex roles of tissue inhibitors of metalloproteinases in cancer. *Oncogene.* (2002). **21**. 2245-2252.

Jinnin M, Ihn H, Mimura Y, Asano Y, Yamane K and Tamaki K. Regulation of fibrogenic/fibrolytic genes by platelet-derived growth factor C, a novel growth factor, in human dermal fibroblasts. *J Cell Physiol.* (2005) **202** 510-517

Joo Y. E., Seo K. S., Kim H. S., Rew J. S., Park C. S., and Kim S. J. Expression of tissue inhibitor of metalloproteinases (TIMPs) in gastric cancer. *Dig Dis Sci* (2000) **45** 114-121

Kawai T, Hiroi S, Torikata C. Expression in lung carcinomas of platelet-derived growth factor and its receptors. *Lab Invest.* (1997) **77** (5) 431-6

Kenny HA, Kaur S, Coussens LM, Lengyel E. The initial steps of ovarian cancer cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin. *J Clin Invest.* (2008) **118** (4) 1367-79

Khan ZE, Wang TC, Cui G, Chi AL, Dimaline R. Transcriptional regulation of the human trefoil factor, TFF1, by gastrin. *Gastroenterology.* (2003) **125** (2) 510-21

Kim J, Yu W, Kovalski K, et al: Requirement for specific proteases in cancer cell intravasation as revealed by a novel semi-quantitative PCR-based assay. *Cell* (1998) **94** 353-362

Kitoh T, Yanai H, Saitoh Y, Nakamura Y, Matsubara Y, Kitoh H, Yoshida T, Okita K. Increased expression of matrix metalloproteinase-7 in invasive early gastric cancer. *J Gastroenterol.* (2004) **39** (5) 434-40

Kleiner DE and Stetler-Stevenson WG. (1999). Matrix metalloproteinases and metastasis. *Cancer Chemother Pharmacol.* **43** (suppl). S42-S51.

Kopin AS, Lee YM, McBride EW, Miller LJ, Lu M, Lin HY, Kolakowski LF Jr, Beinborn M. Expression cloning and characterization of the canine parietal cell gastrin receptor. *Proc Natl Acad Sci U S A.* (1992) **89** (8) 3605-9

Kosako H, Gotoh Y, Matsuda S, Ishikawa M, Nishida E. *Xenopus* MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. *EMBO J* (1992) **11** 2903–2908

Koyama S. Enhanced cell surface expression of matrix metalloproteinases and their inhibitors, and tumor- induced host response in progression of human gastric carcinoma. *Dig Dis Sci* (2004a) **49** 1621-1630

Koyama S. Significance of cell-surface expression of matrix metalloproteinases and their inhibitors on gastric epithelium and infiltrating mucosal lymphocytes in progression of *Helicobacter pylori* associated gastritis. *Scand J Gastroenterol.* (2004b) **39** 1046-1053

Krüger A, Soeltl R, Sopov I, Kopitz C, Arlt M, Magdolen V, Harbeck N, Gänsbacher B and Schmitt M. Hydroxamate-Type Matrix Metalloproteinase Inhibitor Batimastat Promotes Liver Metastasis. *Cancer Research* (2001) **61** 1272-1275

Kubben F. J. G. M., Sier C. F. M., van Duijn W., Griffioen G., Hanemaaijer R., van de Velde C. J. H., van Krieken J. H. J. M, Lamers C. B. H. W., and Verspaget H. W. (2006). Matrix metalloproteinase-2 is a consistent prognostic factor in gastric cancer. *B J Cancer* **94** 1035-1040

Kwak HJ, Park MJ, Cho H, Park CM, Moon SI, Lee HC, Park IC, Kim MS, Rhee CH, Hong SI. Transforming growth factor-beta1 induces tissue inhibitor of metalloproteinase-1 expression via activation of extracellular signal-regulated kinase and Sp1 in human fibrosarcoma cells. *Mol Cancer Res.* (2006) **4** (3) 209-20

Lambert E., Dasse E., Haye B. and Petitfrere E. TIMPs as multifacial proteins. *Clin Rev Oncol Haematol* (2004) **49** 187-198

Leco KJ, Apte SS, Taniguchi GT, Hawkes SP, Khokha R, Schultz GA, Edwards DR.

Murine tissue inhibitor of metalloproteinases-4 (Timp-4): cDNA isolation and expression in adult mouse tissues. *FEBS Lett.* (1997) **401** (2-3) 213-7

Leco KJ, Waterhouse P, Sanchez OH, Gowing KL, Poole AR, Wakeham A, Mak TW, Khokha R. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). *J Clin Invest.* (2001) **108** (6) 817-29

Lee JK, Shin JH, Suh J, Choi IS, Ryu KS, Gwag BJ. Tissue inhibitor of metalloproteinases-3 (TIMP-3) expression is increased during serum deprivation-induced neuronal apoptosis in vitro and in the G93A mouse model of amyotrophic lateral sclerosis: a potential modulator of Fas-mediated apoptosis. *Neurobiol Dis.* (2008) **30** (2) 174-85

Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. *Annu Rev Immunol.* (1998) **16** 137-61

Levi S, Beardshall K, Haddad G, Playford R, Ghosh P, Calam J. Campylobacter pylori and duodenal ulcers: the gastrin link. *Lancet.* (1989) **1** (8648) 1167-8

Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res* (1998) **74** 49–139

Li X, Pontén A, Aase K, Karlsson L, Abramsson A, Uutela M, Bäckström G, Hellström M, Boström H, Li H, Soriano P, Betsholtz C, Heldin CH, Alitalo K, Ostman A, Eriksson U. PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. *Nat Cell Biol.* (2000) **2** (5) 302-9

Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* (1997) **277** 242-245

Liotta LA, Tryggvason K, Garbisa S, et al: Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* (1980) **284** 67-68

Lukashev ME, Werb Z. ECM signalling: orchestrating cell behaviour and misbehaviour. *Trends Cell Biol.* (1998) **8** (11) 437-41

Ma C and Chegini N. Regulation of matrix metalloproteinases (MMPs) and their tissue inhibitors in human myometrial smooth muscle cells by TGF-beta1. *Mol Hum Reprod.* (1999) **5** 950-154

Maity A, Pore N, Lee J, Solomon D, O'Rourke DM. Epidermal growth factor receptor transcriptionally up-regulates vascular endothelial growth factor expression in human glioblastoma cells via a pathway involving phosphatidylinositol 3'-kinase and distinct from that induced by hypoxia. *Cancer Res.* (2000) **60** 5879-5886

Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet.* (1984) **1** (8390) 1311-5

McCaig C, Duval C, Hemers E, Steele I, Pritchard DM, Przemeck S, Dimaline R, Ahmed S, Bodger K, Kerrigan DD, Wang TC, Dockray GJ and Varro A. The role of matrix metalloproteinase-7 in redefining the gastric microenvironment in response to *Helicobacter pylori*. *Gastroenterology*. (2006) **130** 1754-1763

McCawley LJ, Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol*. (2001) **13** (5) 534-40

McColl KE, Gillen D, El-Omar E. The role of gastrin in ulcer pathogenesis. *Baillieres Best Pract Res Clin Gastroenterol*. (2000) **14** (1) 13-26

McKaig BC, McWilliams D, Watson SA and Mahida YR. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol* (2003) **162** 1355-1360

Mellor H and Parker P. The extended protein kinase C superfamily. *Biochem J* (1998) **332** 281–292

Michalopoulos G K & DeFrances M C. Liver regeneration. *Science* (1997) **276** 60-66

Miyake H, Hara I, Gohji K, Yamanaka K, Hara S, Arakawa S, Nakajima M, Kamidono S. Relative expression of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in mouse renal cell carcinoma cells regulates their metastatic potential. *Clin Cancer Res*. (1999) **5** (10) 2824-9

Miyazaki Y, Shinomura Y, Tsutsui S, Zushi S, Higashimoto Y, Kanayama S, Higashiyama S, Taniguchi N, Matsuzawa Y. Gastrin induces heparin-binding epidermal growth factor–like growth factor in rat gastric epithelial cells transfected with gastrin receptor. *Gastroenterology*. (1999) **116** 78-89

Modlin IM, Gilligan CJ, Lawton GP, Tang LH, West AB, Darr U. Gastric carcinoids. The Yale Experience. *Arch Surg*. (1995) **130** (3) 250-5

Mondy JS, Lindner V, Miyashiro JK, Berk BC, Dean RH, Geary RL. Platelet-Derived Growth Factor Ligand and Receptor Expression in Response to Altered Blood Flow *In Vivo*. *Circulation Research*. (1997) **81** 320-327

Mori N, Sato H, Hayashibara T, Senba M, Geleziunas R, Wada A, Hirayama T, Yamamoto N. Helicobacter pylori induces matrix metalloproteinase-9 through activation of nuclear factor kappaB. *Gastroenterology*. (2003) **124** (4) 983-92

Moustakas A, Pardali K, Gaal A and Heldin CH. Mechanisms of TGF-β signalling in regulation of cell growth and differentiation. *Immunol Lett* (2002) **82** 85-91

Moustakas A and Heldin CH. Signalling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer Sci* (2007) **98** 1512-1520

Mozes MM, Böttinger EP, Jacot TA, Kopp JB. Renal expression of fibrotic matrix proteins and of transforming growth factor-beta (TGF-beta) isoforms in TGF-beta transgenic mice. *J Am Soc Nephrol.* (1999) **10** (2) 271-80

Murphy AN, Unsworth EJ, Stetler-Stevenson WG. Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation. *J Cell Physiol.* (1993) **157** (2) 351-8

Nabeshima K, Lane WS, Biswas C: Partial sequencing and characterization of the tumor cell-derived collagenase stimulatory factor. *Arch Biochem Biophys* (1991) **285** 90-96

Nagase H, Woessner JF (1999). Matrix metalloproteinases. *J. Biol Chem.* **274** (31) 21491-21494.

Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S. Molecular cloning and expression of human hepatocyte growth factor. *Nature* (1989) **342** 440–443

Nakamura T, Mizuno S, Matsumoto K, Sawa Y & Matsuda H. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. *J. Clin. Invest.* (2000) **106** 1511–1519

Natali PG, Prat M, Nicotra MR, Bigotti A, Olivero M, Comoglio PM, Di Renzo MF. Overexpression of the met/HGF receptor in renal cell carcinomas. *Int J Cancer.* (1996) **69** (3) 212-7

Noble PJ, Wilde G, White MRH, Pennington SR, Dockray GJ, and Varro A. Stimulation of gastrin-CCK_B receptor promotes migration of gastric AGS cells via multiple paracrine pathways. (2003) *Am J Physiol Gastrointest Liver Physiol.* 284 G75-G84

Normanno N, Bianco C, Strizzi L, Mancino M, Maiello MR, De Luca A, Caponigro F, Salomon DS. The ErbB receptors and their ligands in cancer: An overview. *Curr Drug Targets* (2005) **6** 243-257

Nothnick WB. Disruption of the tissue inhibitor of metalloproteinase-1 gene results in altered reproductive cyclicity and uterine morphology in reproductive-age female mice. *Biol Reprod.* (2000) **63** (3) 905-12

Nothnick WB. Reduction in reproductive lifespan of tissue inhibitor of metalloproteinase 1 (TIMP-1)-deficient female mice. *Reproduction.* (2001a) **122** (6) 923-7

Nothnick WB. Disruption of the tissue inhibitor of metalloproteinase-1 gene in reproductive-age female mice is associated with estrous cycle stage-specific increases in stromelysin messenger RNA expression and activity. *Biol Reprod.* (2001b) **65** (6) 1780-8

Oh J, Sao DW, Diaz T, Wei B, Ward Y, Ray JM, Morioka Y, Shi S, Kitayama H, Tarkahashi C, Noda M and Stetler-Stevenson WG. A review of tissue inhibitor of metalloproteinases-3 (TIMP-3) and experimental analysis of its effect on primary tumor growth. *Cancer Res* (2004) **64** 9062-9

Olivero M, Rizzo M, Madeddu R, Casadio C, Pennacchietti S, Nicotra MR, Prat M, Maggi G, Arena N, Natali PG, Comoglio PM, Di Renzo MF. Overexpression and activation of hepatocyte growth factor/scatter factor in human non-small-cell lung carcinomas. *Br J Cancer.* (1996) **74** (12) 1862-8.

Palmer A, Gavin A C & Nebreda A R. A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *EMBO J.* (1998) **17** 5037-5047

Parikh AA, Liu WB, Fan F, Stoeltzing O, Reinmuth N, Bruns CJ, Bucana CD, Evans DB, Ellis LM. Expression and regulation of the novel vascular endothelial growth factor receptor neuropilin-1 by epidermal growth factor in human pancreatic carcinoma. *Cancer* (2003) **98** 720-729

Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med.* (1991) **325** (16) 1127-31

Pearson G, Robinson F, Beers Gibson T, Xu B, Karandikar M, Berman K and Cobb MH. Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions. *Endocrine Reviews* (2001) **22** (2) 153-183

Peek R M Jr *et al. Helicobacter pylori cagA*$^{+}$ strains and dissociation of gastric epithelial cell proliferation from apoptosis. *J. Natl Cancer Inst.* (1997) 89 863–868

Peek RM Jr, Blaser MJ. Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer.* (2002) **2** (1) 28-37

Peghini PL, Annibale B, Azzoni C, Milione M, Corleto VD, Gibril F, Venzon DJ, Delle Fave G, Bordi C, Jensen RT. Effect of chronic hypergastrinemia on human enterochromaffin-like cells: insights from patients with sporadic gastrinomas. *Gastroenterology.* (2002) **123** (1) 68-85

Piek E, Heldin CH and Dijke PT. Specificity, diversity, and regulation in TGF-ß superfamily signalling. *FASEB.* (1999) **13** 2105-2124

Pohlers D, Huber R, Ukena B, Kinne RW. Expression of platelet-derived growth factors C and D in the synovial membrane of patients with rheumatoid arthritis and osteoarthritis. *Arthritis Rheum.* (2006) **54** (3) 788-94

Pompili VJ, Gordon D, San H, Yang Z, Muller DWM, Nabel GJ, Nabel EG. Expression and function of a recombinant PDGF B gene in porcine arteries. *Arterioscler. Thromb. Vasc. Biol.* (1995) **15** 2254-2264

Poobalarahi F, Baicu CF, and Bradshaw AD. Cardiac myofibroblasts differentiated in 3D culture exhibit distinct changes in collagen I production, processing, and matrix deposition. *Am J Physiol Heart Circ Physiol* (2006) **291** 2924-2932

Porter JF, Shen S, Denhardt DT. Tissue inhibitor of metalloproteinase-1 stimulates proliferation of human cancer cells by inhibiting a metalloproteinase. *Br J Cancer.* (2004) **90** (2) 463-70

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI and West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* (1999) **277** C1-C9

Powell DW, Adegboyega PA, Di Mari JF and Mifflin RC. Epithelial cells and their neighbours I. Role of intestinal myofibroblasts in development, repair, and cancer. *Am J Physiol Gastrointest Liver Physiol* (2005) **289** G2-G7

Powell WC, Knox JD, Navre M, et al: Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. *Cancer Res* (1993) **53** 417-422

Pozzi A, LeVine WF, Gardner HA. Low plasma levels of matrix metalloproteinase 9 permit increased tumor angiogenesis. *Oncogene.* (2002) **21** (2) 272-81

Qureshi HY, Sylvester J, El Mabrouk M and Zafarullah M. TGF-beta-induced expression of tissue inhibitor of metalloproteinases-3 gene in chondrocytes is mediated by extracellular signal-regulated kinase pathway and Sp1 transcription factor. *J. Cell. Physiol.* (2005) **203**. 345-52.

Rabkin R, Fervenza F, Tsao T, Sibley R, Friedlaender M, Hsu F, Lassman C, Hausmann M, Huie P, Schwall RH. Hepatocyte growth factor receptor in acute tubular necrosis. *J Am Soc Nephrol.* (2001) **12** (3) 531-40

Raychowdhury R, Schäfer G, Fleming J, Rosewicz S, Wiedenmann B, Wang TC, Höcker M. Interaction of early growth response protein 1 (Egr-1), specificity protein 1 (Sp1), and cyclic adenosine 3'5'-monophosphate response element binding protein (CREB) at a proximal response element is critical for gastrin-dependent activation of the chromogranin A promoter. *Mol Endocrinol.* (2002) **16** (12) 2802-18

Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P. & Harkonen, M. Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res.* (1997) **57** 1276–1280

Rocha KBF, Soares VA and Viero RM. The Role of Myofibroblasts and Interstitial Fibrosis in the Progression of Membranous Nephropathy. *Renal Failure* (2004) **26** 445 - 451

Rokkas T, Liatsos C, Petridou E, Papatheodorou G, Karameris A, Ladas SD, Raptis SA. Relationship of *Helicobacter pylori* CagA status to gastric cell proliferation and apoptosis. *Dig Dis Sci* (1999) **44** 487–493

Ross R, Glomset J, Kariya B, Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc Natl Acad Sci U S A.* (1974) **71** (4) 1207-10

Santibáñez JF, Iglesias M, Frontelo P, Martínez J, Quintanilla M. Involvement of the Ras/MAPK Signaling Pathway in the Modulation of Urokinase Production and Cellular Invasiveness by Transforming Growth Factor-β_1 in Transformed Keratinocytes. *Biochem Biophys Res Comm.* (2000) **273** 521-527

Sawaoka H, Kawano S, Tsuji S, Tsuji M, Sun W, Gunawan ES, Hori M. *Helicobacter pylori* infection induces cyclooxygenase-2 expression in human gastric mucosa. *Prostaglandins Leukot Essent Fatty Acids.* (1998) **59** 313–316

Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ. MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science.* (1998) **281** (5383) 1668-71

Schonherr E, Jarvelainen HT, Sandell LJ and Wight TN. Effects of platelet-derived growth factor and transforming growth factor-1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J. Biol. Chem.* (1991) **266** 17640-17647

Scotlandi K, Baldini N, Oliviero M, Di Renzo MF, Martano M, Serra M, Manara MC, Comoglio PM, Ferracini R. Expression of Met/hepatocyte growth factor receptor gene and malignant behavior of musculoskeletal tumors. *Am J Pathol.* (1996) **149** (4) 1209-19

Seeland U, Haeuseler C, Hinrichs R, Rosenkranz S, Pfitzner T, Scharffetter-Kochanek K, Böhm M. Myocardial fibrosis in transforming growth factor-beta(1) (TGF-beta(1)) transgenic mice is associated with inhibition of interstitial collagenase. *Eur J Clin Invest.* (2002) **32** (5) 295-303

Sheehan KM, Gulmann C, Eichler GS, Weinstein JN, Barrett HL, Kay EW, Conroy RM, Liotta LA, Petricoin EF 3rd. Signal pathway profiling of epithelial and stromal compartments of colonic carcinoma reveals epithelial-mesenchymal transition. *Oncogene.* (2008) **27** (3) 323-31

Shirin H, Sordillo EM, Oh SH, Yamamoto H, Delohery T, Weinstein IB, Moss SF. *Helicobacter pylori* inhibits the G1 to S transition in AGS gastric epithelial cells. *Cancer Res.* (1999) **59** 2277–2281

Smoot DT, Elliott TB, Verspaget HW, Jones D, Allen CR, Vernon KG, Bremner T, Kidd LC, Kim KS, Groupman JD, Ashktorab H. Influence of *Helicobacter pylori* on reactive oxygen-induced gastric epithelial cell injury. *Carcinogenesis* (2000) **21** 2091-2095

Solcia E, Capella C, Sessa F, Rindi G, Cornaggia M, Riva C, Villani L. Gastric carcinoids and related endocrine growths. *Digestion.* (1986) **35** Suppl 1 3-22

Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* (1995) **19** 183-232

Soriano P. Abnormal kidney development and hematological disorders in PDGF - receptor mutant mice. *Genes Dev.* (1994) **8** 1888-1896

Stepan VM, Tatewaki M, Matsushima M, Dickinson CJ, del Valle J, Todisco A. Gastrin induces c-*fos* gene transcription via multiple signaling pathways. *Am J Physiol Gastrointest Liver Physiol* (1999) **276** G415– 24

Sternlicht MD, Werb Z (2001). How matrix metalloproteinases regulate cell behaviour. *Annu Rev Cell Dev Biol.* **17** 463-516

Stetler-Stevenson WG, Bersch N, Golde DW. Tissue inhibitor of metalloproteinase-2 (TIMP-2) has erythroid-potentiating activity. *FEBS Lett.* (1992) **296** (2) 231-4

Stoker M, Gherardi E, Perryman, M & Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* (1987) **327** 239–242

Sundberg C, Branting M, Gerdin B and Rubin K. Tumor cell and connective tissue cell interactions in human colorectal adenocarcinoma: transfer of platelet-derived growth factor-AB/BB to stromal cells. *Am J Pathol* (1997) **15** 479-492

Sung JJ, Leung WK, Go MY, To KF, Cheng AS, Ng EK, Chan FK. Cyclooxygenase-2 expression in *Helicobacter pylori*-associated premalignant and malignant gastric lesions. *Am J Pathol* (2000) **157** 729–735

Tallquist M and Kazlauskas A. PDGF signalling in cells and mice. *Cyt Growth Factor Rev* (2004) **15** 205-213

Thomas RM, Baybrick JH, Elsayed AM, Sobin LH. Management of patients with gastric carcinoid tumours. *Gastroenterology.* (1995) **108** 296-297.

Thomas RM, Baybick JH, Elsayed AM, Sobin LH. Gastric carcinoids. An immunohistochemical and clinicopathologic study of 104 patients. *Cancer.* (1994) **73** (8) 2053-8.

Thomasset N, Lochter A, Sympson CJ, Lund LR, Williams DR, Behrendtsen O, Werb Z, Bissell MJ. Expression of autoactivated stromelysin-1 in mammary glands of transgenic mice leads to a reactive stroma during early development. *Am J Pathol.* (1998) **153** (2) 457-67

Todisco A, Ramamoorthy S, Witham T, Pausawasdi N, Srinivasan S, Dickinson CJ, Askari FK, Krametter D. Molecular mechanisms for the antiapoptotic action of gastrin. *Am J Physiol Gastrointest Liver Physiol.* (2001) **280** (2) G298-307

Treinies I, Paterson H F, Hooper S, Wilson R & Marshall C J. Activated MEK stimulates expression of AP-1 components independently of phosphatidylinositol 3-

kinase (PI3-kinase) but requires a PI3-kinase signal To stimulate DNA synthesis. *Mol Cell Biol* (1999) **19** 321-329

Treisman R. Regulation of transcription by MAP kinase cascades. *Curr Opin Cell Biol* (1996) **8** 205-215

Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nat Rev Cancer.* (2002) **2** (4) 289-300

Tsutsui S, Shinomura Y, Higashiyama S, Higashimoto Y, Miyazaki Y, et al. Induction of heparin binding epidermal growth factor-like growth factor and amphiregulin mRNAs by gastrin in the rat stomach. *Biochem Biophys Res Commun* (1997) **235** 520-23

Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T, Kitamura N. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature.* (1995) **373** (6516) 702-5

Uemura N, Mukai T, Okamoto S, Yamaguchi S, Mashiba H, Taniyama K, Sasaki N, Haruma K, Sumii K, Kajiyama G. Effect of Helicobacter pylori eradication on subsequent development of cancer after endoscopic resection of early gastric cancer. *Cancer Epidemiol Biomarkers Prev.* (1997) **6** (8) 639-42.

Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N and Schlemper RJ (2001). *Helicobacter pylori* Infection and the Development of Gastric Cancer. *New Engl J Med* **345** 784-789

Valente P, Fassina G, Melchiori A, Masiello L, Cilli M, Vacca A, Onisto M, Santi L, Stetler-Stevenson WG, Albini A. TIMP-2 over-expression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis. *Int J Cancer.* (1998) **75** (2) 246-53

Varro A, Noble PJ, Wroblewski LE, Bishop L, Dockray GJ. Gastrin-cholecystokinin$_B$ receptor expression in AGS cells is associated with direct inhibition and indirect stimulation of cell proliferation via paracrine activation of the epidermal growth factor receptor. *Gut* (2002a) **50** 827-833

Varro A, Hemers E, Archer D, Pagliocca A, Haigh C, Ahmed S, Dimaline R, Dockray GJ.
Identification of plasminogen activator inhibitor-2 as a gastrin-regulated gene: Role of Rho GTPase and menin. *Gastroenterology.* (2002b) **123**(1) 271-80

Varro A, Kenny S, Hemers E, McCaig C, Przemeck S, Wang TC, Bodger K, Pritchard DM.
Increased gastric expression of MMP-7 in hypergastrinemia and significance for epithelial-mesenchymal signaling. *Am J Physiol Gastrointest Liver Physiol.* (2007) **292** (4) G1133-40

Vignaud JM, Allam M, Martinet N, Pech M, Plenat F, Martinet Y. Presence of platelet-derived growth factor in normal and fibrotic lung is specifically associated

with interstitial macrophages, while both interstitial macrophages and alveolar epithelial cells express the c-sis proto-oncogene. *Am J Respir Cell Mol Biol.* (1991) **5** (6) 531-8

Voorzanger-Rousselot N, Juillet F, Mareau E, Zimmermann J, Kalebic T, Garnero P. Association of 12 serum biochemical markers of angiogenesis, tumour invasion and bone turnover with bone metastases from breast cancer: a crossectional and longitudinal evaluation. *Br J Cancer.* (2006) **95** (4) 506-14

Wagner, S. *et al.* Regulation of gastric epithelial cell growth by *Helicobacter pylori*: evidence for a major role of apoptosis. *Gastroenterology* (1997) **113** 1836–1847

Walsh JH (1994) Gastrin. In Walsh JH, Dockray GJ (eds) Gut peptides. Raven Press, New York 75-121.

Wang TC, Koh TJ, Varro A, Cahill RJ, Dangler CA, Fox JG, Dockray GJ. Processing and proliferative effects of human progastrin in transgenic mice. *J Clin Invest.* (1996) **98** (8) 1918-29.

Wang TC, Dangler CA, Chen D, Goldenring JR, Koh T, Raychowdhury R, Coffey RJ, Ito S, Varro A, Dockray GJ, Fox JG. Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. *Gastroenterology.* (2000) **118** (1) 36-47.

Watanabe T, Tada M, Nagai H, Sasaki S, Nakao M. Helicobacter pylori infection induces gastric cancer in mongolian gerbils. *Gastroenterology.* (1998) **115** (3) 642-8

Watson F, Kiernan RS, Deavall DG, Varro A, Dimaline R. Transcriptional activation of the rat vesicular monoamine transporter 2 promoter in gastric epithelial cells: regulation by gastrin. *J Biol Chem.* (2001) **276** (10) 7661-71

Weber BH, Vogt G, Pruett RC, Stöhr H, Felbor U. Mutations in the tissue inhibitor of metalloproteinases-3 (TIMP3) in patients with Sorsby's fundus dystrophy. *Nat Genet.* (1994) **8** (4) 352-6

Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. *Cell.* (1997) **91** (4) 439-42

Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J & Davis RJ. A mammalian scaffold complex that selectively mediates MAP kinase activation. *Science.* (1999) **281** 1671-1674

Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM and Borok Z. Induction of Epithelial-Mesenchymal Transition in Alveolar Epithelial Cells by Transforming Growth Factor-ß1: Potential Role in Idiopathic Pulmonary Fibrosis. *Am J pathol* (2005) **166** 1321-1332

Wingfield PT, Sax JK, Stahl SJ, Kaufman J, Palmer I, Chung V, Corcoran ML, Kleiner DE, Stetler-Stevenson WG. Biophysical and functional characterization of full-length, recombinant human tissue inhibitor of metalloproteinases-2 (TIMP-2)

produced in Escherichia coli. Comparison of wild type and amino-terminal alanine appended variant with implications for the mechanism of TIMP functions. *J Biol Chem.* (1999) **274** (30) 21362-8

Woessner JF. That impish TIMP: the tissue inhibitor of metalloproteinase-3. *J Clin Invest.* (2001) **108**. 799-800.

Wroblewski LE, Noble PJ, Pagliocca A, Pritchard DM, Hart CA, Campbell F, Dodson AR, Dockray GJ and Varro A. Stimulation of MMP-7 (matrilysin) by *Helicobacter pylori* in human gastric epithelial cells: role in epithelial cell migration. *J. Cell. Science.* (2003). **116** 3017-3026.

Wroblewski L. Pritchard DM., Carter S., and Varro A. (2002) Gastrin stimulated gastric epithelial cell invasion: the role and mechanism of increased matrix metalloproteinase 9 expression. *Biochem J.* **365**. 873-879.

Wu KC, Jackson LM, Galvin AM, Gray T, Hawkey CJ and Mahida YR. Phenotypic and functional characterisation of myofibroblasts, macrophages and lymphocytes migrating out of the human gastric lamina propria following the loss of epithelial cells. *Gut.* (1999) **44** 323-330

Xia Y, Wu Z, Su B, Murray B and Karin M. JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension. *Genes Dev.* (1998) **12** 3369-3381

Xia Z, Dickens M, Raingeaud J, Davis R J and Greenberg M E. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* (1995) **270** 1326-1331

Yi ES, Lee H, Yin S, Piguet P, Sarosi I, Kaufmann S, Tarpley J, Wang NS and Ulich TR. Platelet-derived growth factor causes pulmonary cell proliferation and collagen deposition in vivo. *Am J Pathol* (1996) **149** 539-548

Yoshikawa T, Tsuburaya A, Kobayashi O, Sairenji M, Motohashi H, Yanoma S and Noguchi Y. Intratumoural concentration of tissue inhibitor of matrix metalloproteinase 1 in patients with gastric carcinoma: a new biomarker and its impact on survival. *Cancer* (2001) **91** 1739-1744

Young MR, Colburn NH. Fra-1 a target for cancer prevention or intervention. *Gene* (2006) **379** 1–11

Yu WH, Yu S, Meng Q, Brew K, Woessner JF Jr. TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. *J Biol Chem.* (2000) **275** (40) 31226-32

Zhai Y, Hotary KB, Nan B, Bosch FX, Muñoz N, Weiss SJ, Cho KR. Expression of membrane type 1 matrix metalloproteinase is associated with cervical carcinoma progression and invasion. *Cancer Res.* (2005) **65** (15) 6543-50

Zheng XY, Zhang JZ, Tu P, Ma SQ. Expression of platelet-derived growth factor B-chain and platelet-derived growth factor beta-receptor in fibroblasts of scleroderma. *J Dermatol Sci.* (1998) **18** (2) 90-7

8.1 Appendix 1

8.1.1 Restriction enzymes used and reaction conditions

Enzyme	Company	Buffer	Temperature (°C)	BSA Y/N
HindIII	Sigma	NEB 2	37	N
NheI	NEB	NEB 2	37	Y
XhoI	Sigma	NEB 2	37	Y
KpnI	Sigma	NEB 1	37	Y
BglII	Sigma	Sigma SM/ NEB 3	37	N
SalI	Sigma	Sigma SH	37	Y
BglI	Sigma	Sigma SH/NEB 3	37	N
AscI	NEB	NEB 4	37	N
NsiI	NEB	NEB 3	37	N
BsaI	NEB	NEB 3	50	N
SmaI	Sigma	NEB 4	25	N

8.1.2 Double restriction enzymes digest reaction conditions.

Enzyme 1	Enzyme 2	Buffer	Temperature (°C)	BSA (Y/N)
BglII	SalI	Sigma SH	37	N
BglII	XhoI	Sigma SH	37	N
HindIII	SalI	NEB 2	37	N
HindIII	XhoI	NEB 2	37	Y
AscI	NheI	NEB 4	37	Y
NheI	NsiI	NEB 2	37	N
BsaI	NheI	NEB 4	37 for 1h 50 for 1h	N
BglII	KpnI	NEB 2	37	Y

8.2 Appendix 2

8.2.1 Composition of QIAGEN buffers used for purification of plasmid DNA and gel elution of DNA

QIAGEN

Buffer P1

50mM Tris-Cl pH8, 10mM Na_2EDTA in 100µg/ml RNase A.

Buffer P2

200mM NaOH, 1% SDS (w/v).

Buffer P3

1M potassium acetate pH5.5.

Buffer QBT

750mM NaCl, 50mM MOPS pH7, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v).

Buffer QC

1M NaCl, 50mM MOPS pH7, 15% isopropanol (v/v).

Buffer QF

1.25M NaCl, 50mM Tris-Cl pH8.5, 15% isopropanol (v/v).

Buffer EB

10 mM Tris-Cl, pH 8.5

8.3 Appendix 3

Q-PCR primer (F & R) and probe (T) sequences

Mouse

TIMP-1

F: 5'-CCAGAACCGCAGTGAAGAGTT

R: 5'-AAGCTGCAGGCACTGATGTG

T: 5'-CTCATCACGGGCCGCCTAAGGA

TIMP-3

F: 5'-GGCACTCTGGTCTACACTATTAAGCA

R: 5'-CAGAGGCTTCCGAGAGAATGT

T: 5'-CGAGGCTTCAGTAAGATGCCCCACGT

TIMP-4

F: 5'-GCCACTCGGCTCTAGTGATACG

R: 5'-GAGTGTCAGGGTCTTTGC

T: 5'-CCAAAATATCCAGTGAGAAGGTAGTCCCTGCC

Human

TIMP-1

F: 5'-GCTGACATCCGGTTCGTCTAC

R: 5'- GTTGTGGGACCTGTGGAAGTATC

T: 5'- CCCCCGCCATGGAGAGTGTCTG

TIMP-2

F: 5'-CCTGAACCACAGGTACCAGATG

R: 5'-AGGAGATGTAGCGGATCA

T: 5'-AGTGCAAGATCACGCGCTGCCC

TIMP-3

F: 5'-TTCGGTTACCCTGGCTACCA

R: 5'-GCAGTAGCCGCCCTTCTG

T: 5'-TCCAAACACTACGCCTGCATCCGG

TIMP-4

F: 5'-CACCCTCAGCAGCACATCTG

R: 5'-GGCCGGAACTACCTTCTCACT

T: 5'-ACTCGGCACTTGTGATTCGGGCC

8.4 Appendix 4

8.4.1 Primary Antibodies used for Western Blotting

Antibody	Type	Dilution	company
Timp-1 6D11	Mouse anti human	1:100	RDI
Timp-1 AB8112	Rabbit anti human	1:1000	Chemicon
Timp-2 4H11	Mouse anti human	1:1000	RDI
Timp-2 AB19078	Rabbit anti human	1:200	RDI
Timp-2 AB8107	Rabbit anti human	1:1000	Chemicon
Timp-3 C1	Mouse anti human	1:1000	RDI
Timp-3 AB802	Rabbit anti human	1:2000	Chemicon
Timp-3 MAB3318	Mouse anti human	1:1000	Chemicon
Timp-4 AB816	Rabbit anti human	1:1000	Chemicon

8.4.2 Secondary Antibodies

Antibody	Dilution	company
Goat Anti Mouse	1:5000	Sigma
Goat Anti Rabbit	1:10,000	Pierce

8.5 Appendix 5

Antibodies used for Immunocytochemistry

Antibody	Type	Dilution	company
Timp-1 C1	Mouse anti human	1:100	RDI
Timp-1 6D11	Mouse anti human	1:100	RDI
Timp-2 4H11	Mouse anti human	1:200	RDI
Timp-3 C1	Mouse anti human	1:20	RDI
Timp-3 AB802	Rabbit anti human	1:200	Chemicon
Timp-3 MAB3318	Mouse anti human	1:100	Chemicon
Timp-3 SC-6838	Goat anti human	1:100	Santa Cruz
Timp-4 RB-1542-R7	Rabbit anti human	Neat	Stratech
Timp-4 AB816	Rabbit anti human	1:200	Chemicon
anti-fos 554156	Mouse anti human	1:250	PharMingen

8.6 Appendix 6

Map of pGL4.10 luciferase vector

(obtained from www.promega.com)